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Epidermal growth factor receptor inhibition downregulates *Helicobacter pylori*-induced epithelial inflammatory responses, DNA damage, and gastric carcinogenesis

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Abstract

Objective—Gastric cancer is the third leading cause of cancer death worldwide and infection by *Helicobacter pylori* is the strongest risk factor. We have reported increased epidermal growth factor receptor (EGFR) phosphorylation in the *H. pylori*-induced human carcinogenesis cascade, and association with DNA damage. Our goal was to determine the role of EGFR activation in gastric carcinogenesis.

Design—We evaluated gefitinib, a specific EGFR inhibitor, in chemoprevention of *H. pylori*-induced gastric inflammation and cancer development. Mice with genetically-targeted epithelial cell-specific deletion of *Egfr* (*Egfr*^{epi} mice) were also used.

Results—In C57BL/6 mice, gefitinib decreased *Cxcl1* and *Cxcl2* expression by gastric epithelial cells, myeloperoxidase-positive inflammatory cells in the mucosa, and epithelial DNA damage induced by *H. pylori* infection. Similar reductions in chemokines, inflammatory cells, and DNA damage occurred in infected *Egfr*^{epi} versus *Egfr*^{fl/fl} control mice. In *H. pylori*-infected transgenic

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Contributors JCS performed the experiments, analysed the data, and drafted the manuscript. MA, TGV, GS, JR-G, AGD, and LEW performed experiments. MBP analysed and scored all of the tissue sections. DPB designed and generated epithelial-specific knockout mice. RMP provided funding and commented on the manuscript. APG designed experiments and revised the manuscript. KTW designed the experiments, analysed the data, supervised the studies, obtained funding, and also wrote the paper.

insulin–gastrin (INS-GAS) mice and gerbils, gefitinib treatment markedly reduced dysplasia and carcinoma. Gefitinib blocked *H. pylori*-induced activation of mitogen-activated protein kinase 1/3 (MAPK1/3) and activator protein 1 in gastric epithelial cells, resulting in inhibition of chemokine synthesis. MAPK1/3 phosphorylation and JUN activation was reduced in gastric tissues from infected wild-type and INS-GAS mice treated with gefitinib and in primary epithelial cells from *Egfr^{epi}* versus *Egfr^{fl/fl}* mice. Epithelial EGFR activation persisted in humans and mice after *H. pylori* eradication, and gefitinib reduced gastric carcinoma in INS-GAS mice treated with antibiotics.

Conclusions—These findings suggest that epithelial EGFR inhibition represents a potential strategy to prevent development of gastric carcinoma in *H. pylori*-infected individuals.

Keywords

EGFR; *Helicobacter pylori*; gastric cancer; chemokines; chemoprevention

Introduction

Infection with *Helicobacter pylori* is directly associated with development of gastric cancer in humans[1], the third leading cause of cancer death worldwide (globocan.iarc.fr). While systematic antibiotic-based eradication has been proposed[2], treatment failure is common, and high rates of recurrence and/or recrudescence occur in developing countries[3, 4]. Furthermore, the benefit of *H. pylori* eradication is uncertain once precancerous lesions develop[5]. Thus, alternative strategies are needed to reduce gastric cancer incidence.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein belonging to the receptor tyrosine kinase group[6]. EGFR activation enhances cell growth, proliferation, differentiation, and wound healing, but has been implicated in development of malignancies, including gastric cancer[7, 8]. EGFR overexpression is observed in 27-44% of gastric cancer cases and is associated with worse outcomes[7, 9]. Our group previously reported that EGFR transactivation (phosphorylation) is linked to DNA damage in gastric epithelial cells (GECs) after infection with *H. pylori*, both in cell lines and in tissues, and that epithelial levels of activated EGFR are increased in human gastritis and atrophic gastritis, but not in the later stages of the cancer cascade[10]. Importantly, levels of phosphorylated EGFR (pEGFR) were upregulated in baseline gastric biopsies from subjects in a long-term cohort in Colombia that later progressed to intestinal metaplasia or dysplasia as compared to non-progressors[10]. These data suggest that EGFR activation is involved in events leading to gastric cancer. Gefitinib, an inhibitor of EGFR, has been used in the treatment of a variety of cancers and has shown efficacy in human non-small cell lung cancer and proximal gastric cancer[11, 12], and as a chemopreventive agent for lung and breast cancer in animal models[13, 14].

Herein, we show that gefitinib treatment of *H. pylori*-infected mice and gerbils attenuated EGFR phosphorylation, the pro-inflammatory response of GECs, DNA damage, and development of inflammation, dysplasia, and cancer. Epithelial-specific gene knockout of *Egfr* also decreased innate immune response of GECs, gastric inflammation, and DNA damage. The protective effect of gefitinib was linked to inhibition of mitogen-activated

protein kinase 1/3 (MAPK1/3)-activator protein 1 (AP-1) signaling. Our findings indicate a critical role for EGFR signaling in gastric inflammation and carcinogenesis, and that inhibition of EGFR may serve as a strategy for decreasing cancer risk.

Methods

Cells and bacteria

Mouse conditionally immortalized stomach epithelial (ImSt) were used as described[10, 15]. AGS cells were from ATCC. Cells were pre-treated with 10 μ M gefitinib, PD 98059, or SR 11302, for 1 h and infected with *H. pylori* PMSS1, 7.13, or 60190 and its isogenic *cagA* and *vacA* mutants, at a multiplicity of infection (MOI) of 10-100[10].

Generation of *Egfr^{epi}* mice

Egfr^{tm1Dwt} mice were crossed to a Tg(Foxa3-cre)1Khk transgene-expressing mouse strain[16, 17] generating *Egfr^{fl/fl};Foxa3^{cre/+}* (*Egfr^{epi}*) mice. Littermate *Egfr^{fl/fl}* and *Egfr^{epi}* mice were used for experiments.

Animals and infections

Mice and gerbils were fed a defined rodent diet (AIN-76A, BioServ), supplemented or not with 200 ppm gefitinib[18], one week before infection and for the duration of experiments. C57BL/6, *Egfr^{fl/fl}*, *Egfr^{epi}*, and transgenic insulin–gastrin (INS–GAS) mice were infected with 1×10^9 CFU of *H. pylori* PMSS1 a *vacA s2/m2* strain [15]; Mongolian gerbils were infected with 2×10^9 CFU of *H. pylori* 7.13 [19] a VacA-negative strain [20]. Colonization of all included animals was verified by culture[19, 21].

Isolation of epithelial cells from gastric tissue

GECs were isolated by dissociation and dispersion[15].

Western blotting

Proteins from cell lines and tissues were analysed as described[10]. Antibodies are summarized in Supplementary Methods.

Immunohistochemistry and imaging analysis

See Supplementary Methods.

Flow cytometry

See Supplementary Methods.

Chemokine and cytokine analysis

Gastric tissues were lysed and processed using a Mouse Milliplex kit (Millipore) and analysed on a Luminex FlexMap 3D machine[17]. CXCL1 was also measured using the DuoSet ELISA Mouse CXCL1 kit (R&D Systems).

mRNA analysis

Expression of *Cxcl1*, *Cxcl2*, *Il1b*, and *CXCL8* was assayed by real-time PCR[15, 17].

Culture of primary gastric epithelial cell monolayers

See Supplementary Methods.

Statistical analysis

Results are expressed as mean $\pm$ SEM. For multiple groups, comparisons were performed using the Kruskal-Wallis nonparametric multiple comparison test followed by paired comparisons using the Mann-Whitney test. Contingency analysis was performed using the χ^2 test.

Results

Gefitinib effectively reduces EGFR activation and DNA damage in response to *H. pylori* in vivo

EGFR activation in gastric tissues has been demonstrated as early as 24 h after *H. pylori* infection[22]. Accordingly, EGFR was phosphorylated at tyrosine residue 1068 in mice infected for 1, 2, or 7 days with PMSS1 (supplementary figure S1 and figure 1A). EGFR activation in GECs was blocked when the diet was supplemented with gefitinib (figure 1A). Colonization density was increased in gefitinib-treated versus control mice (supplementary figure S2A). Chronic inhibition was confirmed by immunohistochemistry showing a marked attenuation of pEGFR in gastric tissues from mice infected with *H. pylori* strain PMSS1 for one month and treated with gefitinib compared to animals receiving the control diet (figure 1B).

There was increased phosphorylation of H2AFX at serine 139, a marker of DNA damage[23], when assessed by flow cytometry in GECs isolated from *H. pylori*-infected mice versus uninfected animals (figure 1C). Significant reduction of pH2AFX-positive GECs was observed in infected mice maintained on the gefitinib-supplemented diet (figure 1C). A similar reduction in GECs positive for pH2AFX was observed by immunohistochemistry, quantified by image analysis, in infected mice on the gefitinib diet (figure 1D and 1E).

EGFR inhibition leads to a reduction on *H. pylori*-induced inflammatory response

We directly evaluated levels of inflammatory cell infiltration in the gastric mucosa of *H. pylori*-infected mice by immunohistochemistry for MPO, an enzyme abundantly expressed in polymorphonuclear cells (PMNs). The number of MPO-positive cells was increased in PMSS1-infected mice as compared to uninfected controls (figure 2A), and inhibition of EGFR activation by gefitinib significantly reduced the number of infiltrating cells as quantified by image analysis (figure 2A and 2B). We then assessed chemokines and cytokines produced in gastric tissues by Luminex-based multiplex protein analysis. In mice infected with *H. pylori*, there were increased concentrations of the chemokines CXCL1, CXCL2, and CCL5; the macrophage-derived cytokines CSF3 and IL-1 β ; and the prototype Th17 cytokine, IL-17 (figure 2C). The induction of each of these pro-inflammatory

parameters was decreased with gefitinib treatment in infected animals (figure 2C). Additional targets in the multiplex assay not significantly affected by gefitinib treatment are in supplementary table 1.

To confirm the role of epithelial EGFR as a critical mediator of *H. pylori*-induced gastric inflammation, we used *Egfr^{epi}* mice. Western blot analysis demonstrated loss of EGFR in GECs isolated from *Egfr^{epi}* mice, when compared to *Egfr^{fl/fl}* mice (figure 3A). This was confirmed by immunohistochemistry for pEGFR, showing epithelial staining in the tissues from PMSS1-infected *Egfr^{fl/fl}* mice and a marked reduction in infected *Egfr^{epi}* mice (figure 3B). There was no difference in colonization between *Egfr^{fl/fl}* and *Egfr^{epi}* mice (supplementary figure S2B). *Egfr^{fl/fl}* mice infected with *H. pylori* showed more MPO-positive cells than uninfected animals (figure 3C) and a significant reduction of MPO staining was observed in *H. pylori*-infected *Egfr^{epi}* mice (figure 3C). Concomitantly, levels of *Cxcl1* and *Cxcl2* mRNA in isolated GECs from infected animals were reduced in *Egfr^{epi}* mice compared to *Egfr^{fl/fl}* mice (figure 3D). DNA damage in gastric tissues of infected mice was also significantly reduced in *Egfr^{epi}* mice compared to *Egfr^{fl/fl}* mice (figure 3E).

Gefitinib decreases development of neoplastic lesions and inflammation in INS-GAS mice infected with *H. pylori*

To determine the effect of EGFR on *H. pylori*-induced gastric carcinogenesis, hypergastrinemic INS-GAS mice that can develop dysplasia and carcinoma with *H. pylori* infection[15] were given the control or gefitinib-supplemented diet during a 2-month infection with *H. pylori* strain PMSS1. Colonization was increased in INS-GAS mice on gefitinib (supplementary figure S2C). As shown in figure 4A, on the control diet, 79% developed dysplasia, and 41% developed intramucosal carcinoma; when mice were given the gefitinib diet, there were significant decreases in development of dysplasia (to 55%) and carcinoma (to 3%). Extent of dysplasia and carcinoma as a percentage of the gastric mucosa was increased with infection and significantly attenuated by gefitinib (figure 4B). The modest effects of gefitinib on uninfected mice in figure 4A and 4B were not statistically significant. Figure 4C depicts H&E staining of the gastric mucosa of an infected INS-GAS mouse on the control diet, showing intramucosal carcinoma, with irregular and angulated glands, infiltration of the lamina propria by tumor cells, and desmoplastic stroma; in contrast, the gefitinib-treated mouse shown exhibited only low-grade dysplasia. When INS-GAS mice on the gefitinib diet were compared to those on the control diet, there were decreases in gastric tissue MPO-positive cells (figure 4D), *Cxcl1* and *Cxcl2* mRNA levels (figure 4E) and CXCL1 protein levels (figure 4F) in isolated GECs, and pH2AFX positive cells in the GECs *in situ* (figure 4G). In infected INS-GAS mice there was an increase in infiltrating T cells, detected by CD3 immunostaining, which was decreased by gefitinib (supplementary figure S3).

Gefitinib treatment reduces DNA damage and gastric carcinogenesis in Mongolian gerbils infected with *H. pylori*

To establish the role of EGFR activation in a model of *H. pylori*-induced cancer, we used Mongolian gerbils infected with *H. pylori* 7.13, known to induce gastric carcinoma in these animals[15, 24]. There was increased *H. pylori* colonization in gefitinib-treated gerbils after

8, but not 12 weeks of infection (supplementary figure S2D-E). Figure 5A illustrates representative H&E staining of the gastric antrum of infected gerbils. Gerbils on the control diet exhibited invasive adenocarcinoma, characterized by irregular and angulated glands infiltrating the submucosa. In contrast, the depicted example from a gerbil on the gefitinib diet shows only low-grade dysplasia characterized by elongated, branched, irregular glands without infiltration of the submucosa. On the control diet, infection resulted in invasive gastric adenocarcinoma in 60% and 87.5% of gerbils at 8-weeks and 12-weeks post-infection, respectively (figure 5B). Administration of gefitinib resulted in a significant decrease in gastric cancer, to 31.25% and 62.5%, at these time points (figure 5B).

Infection of gerbils by *H. pylori* was also associated with increased MPO-positive cells in the gastric mucosa (figure 5C), which was decreased in animals treated with gefitinib (figure 5C). In parallel, a significant increase in mRNA expression of the pro-inflammatory markers *Cxcl1* and *Il1b* was observed in GECs from infected gerbils on the control diet, and these mRNA levels were significantly reduced by gefitinib treatment (figure 5D). Moreover, there was a significant decrease in pH2AFX-positive cells in infected gerbils on the gefitinib versus control diet (figure 5E and 5F).

To evaluate gefitinib after *H. pylori* infection was established, we started treatment 2 weeks post-infection (supplementary figure S4A). Gefitinib treatment either before, or after infection resulted in the same reduction in gastric adenocarcinoma (supplementary figure S4B) and a similar decrease in MPO-positive cells (supplementary figure S4C).

DNA damage and chemokine production in GECs is mediated by EGFR-dependent ERK/AP-1 activation

To recapitulate effects of EGFR inhibition *in vitro*, we infected ImSt cells with *H. pylori* strains PMSS1 or 7.13 and showed that gefitinib efficiently inhibits EGFR activation (supplementary figure S5A). mRNA levels of *Cxcl1* and *Cxcl2* were induced in response to both *H. pylori* strains (figure 6A and 6B), and gefitinib significantly decreased expression of these genes (figure 6A and 6B). We also assessed CXCL1 secretion by ELISA, and found that levels of this chemokine were upregulated in response to *H. pylori* and markedly inhibited when cells were treated with gefitinib (figure 6C). When DNA damage was assessed by flow cytometry, there was an increased percentage of pH2AFX-positive cells after infection with either *H. pylori* strain, and a significant decrease in the cells treated with gefitinib (figure 6D).

In the human cell line AGS, expression of the gene *CXCL8*, also known as IL-8, the human homologue of the murine *Cxcl1*, was induced in AGS cells infected with *H. pylori* PMSS1 or 7.13 (figure 7A). We confirmed the reduction of pEGFR by gefitinib in AGS cells (supplementary figure S5B). Also, EGFR activation and *CXCL8* expression occurred upon infection with lower bacterial densities (supplementary figure S6E-F). There was significant inhibition of *CXCL8* mRNA expression in infected cells treated with gefitinib (figure 7A; supplementary figure S6F), as well as with the MAPK1/3 (ERK1/2) inhibitor, PD 98059, or the AP-1 inhibitor, SR 11302 (figure 7A).

Consistent with these results, we found that infection of AGS cells with *H. pylori* induced JUN translocation from the cytoplasm to the nucleus, indicating activation of AP-1 (figure 7B); nuclear translocation of JUN was markedly reduced in cells with EGFR or MAPK1/3 inhibition (figure 7B); as a positive control, SR 11302 blocked JUN activation (figure 7B). Cytoplasmic JUN was not reduced by the inhibitors, when assessed as a control (supplementary figure S6G). There was induction of NF- κ B activation, by Western blot analysis of phosphorylated RELA (phospho-p65), with infection by *H. pylori* PMSS1 or 7.13, but there was no decrease in NF- κ B activation with EGFR inhibition (figure 7C). NF- κ B activation was not altered in gefitinib-treated cells after infection with *H. pylori* (supplementary figure S6H). This indicates that activation of JUN/AP-1, but not NF- κ B, requires signaling involving EGFR and MAPK1/3. In addition, gefitinib reduced MAPK1/3 phosphorylation in response to infection with *H. pylori* PMSS1 or 7.13 in AGS cells (figure 7C), demonstrating that MAPK1/3 activation by *H. pylori* is mediated through EGFR.

Gefitinib may target other receptor kinases, like RIPK2[25]. *H. pylori* induced significant RIPK2 phosphorylation in AGS cells (supplementary figure S6A)[26]. However, gefitinib did not affect pRIPK2 in infected cells (supplementary figure S6A-B).

CagA and VacA have been reported to modulate EGFR activation [27, 28]. However, similar levels of EGFR activation were induced by WT, or *cagA* and *vacA* isogenic mutants of *H. pylori* (supplementary figure S6C-D).

MAPK1/3 phosphorylation was increased *in vivo* in infected C57BL/6 and INS-GAS mice and reduced by gefitinib, when assessed by western blotting and densitometry (supplementary figure S7A-D). By immunohistochemistry, pMAPK1/3 levels were attenuated in gastric tissues of *H. pylori*-infected mice on gefitinib (figure 7D and E), along with pJUN levels (figure 7D and E; supplementary figure S8A-B). Reduced pEGFR levels with gefitinib were confirmed in INS-GAS mice by immunohistochemistry (figure 7E).

MAPK1/3-JUN signaling pathway activation and chemokine production are decreased in cultures of GEC isolated from *Egfr^{epi}* mice

Using immunofluorescence, we confirmed that *H. pylori* stimulation *ex vivo* induced a marked increase in pEGFR in two-dimensional monolayers of primary GEC isolated from *Egfr^{fl/fl}* mice, and this activation was not observed in monolayers from *Egfr^{epi}* mice (figure 8A). *H. pylori* also induced marked MAPK1/3 and JUN activation in cells from *Egfr^{fl/fl}* mice, which was prevented in the cells from *Egfr^{epi}* mice (figure 8A and supplementary figure S9). *Cxcl1* and *Cxcl2* expression was significantly increased in *Egfr^{fl/fl}* GEC after *H. pylori* infection, which did not occur in GEC from *Egfr^{epi}* mice (figure 8B).

EGFR is still activated after *H. pylori* eradication in humans

EGFR activation was assessed by immunohistochemistry in gastric tissues from a cohort of *H. pylori*-infected Colombian patients pre- and post-antibiotic eradication therapy. Variable levels of pEGFR staining were observed in pre-treatment biopsies (figure 9A). EGFR activation was not attenuated after *H. pylori* eradication (figure 9A). Epithelial and immune cell staining for pEGFR was observed in pre-treatment biopsies (figure 9B, left); there was a

loss of inflammation post-treatment, but pEGFR was still present in epithelial cells (figure 9B, right).

Gefitinib treatment reduces development of neoplastic lesions even after *H. pylori* eradication

To determine the effect of gefitinib after *H. pylori* eradication, INS-GAS mice were infected for 4 weeks, and then antibiotics were administered for 1 week, followed by 3 weeks of gefitinib. All mice were verified to have elimination of *H. pylori* infection by antibiotics. As shown in figure 9C, intramucosal carcinoma was significantly reduced by gefitinib treatment during the last 3 weeks of infection; the incidence of intramucosal carcinoma went from 50% in the untreated group to 8.3% in gefitinib-treated animals. Antibiotics reduced the incidence of carcinoma to 23%, but this did not reach statistical significance. Gefitinib treatment after antibiotics showed a further benefit; none of the mice in this group developed intramucosal carcinoma, and 80% showed no signs of dysplasia, a significant reduction versus both untreated and antibiotic-treated animals. Figure 9D illustrates representative H&E staining of the gastric mucosa of infected mice in each group.

pEGFR was evaluated by immunohistochemistry and the intensity of staining was quantified (figure 9E). Abundant pEGFR staining in INS-GAS mice infected with *H. pylori* was detected and was reduced in mice treated with gefitinib during the last 2 weeks of infection. Antibiotic treatment did not reduce pEGFR staining compared to the untreated group. Gefitinib treatment after antibiotics also led to less pEGFR compared to the untreated group. Representative images of pEGFR staining are shown in figure 9F.

Discussion

H. pylori infection is accompanied by the potent induction of both innate and adaptive mucosal responses, which play a fundamental role in development of the chronic active inflammation that characterizes the disease phenotype[29, 30]. The dysregulated synthesis of immune effectors, including reactive oxygen or nitrogen species, also has deleterious and pro-carcinogenic effects on the gastric epithelium[31, 32]. In the present report, we have implicated activation of epithelial EGFR in gastric inflammation, chemokine expression, DNA damage, and development of cancerous lesions. Our findings implicate MAPK1/3- and AP-1-mediated chemokine production by the gastric epithelium that leads to recruitment of immune cells, and thus induction and maintenance of inflammation during *H. pylori* infection. Our data showing that EGFR inhibition by gefitinib either as pre-treatment or when is administered after the infection is established led to decreased inflammatory cells in the infected mucosa, phosphorylation of H2AFX, and gastric dysplasia and carcinoma, have been obtained in both mice and gerbils. The inhibitory effect of gefitinib on *H. pylori*-induced MAPK1/3 phosphorylation and chemokine expression was recapitulated in human GECs, emphasizing that this mechanism is not species-dependent. EGFR inhibitors are in current use in patients with breast and lung cancer; in the case of gastric cancer the efficacy of these agents appears to be more limited, but is under continued investigation [33, 34]. Importantly, our studies were not designed to investigate gefitinib as an agent to treat gastric

cancer, but rather, suggest that it may prove useful to prevent development of neoplastic lesions by blocking inflammation-related DNA damage.

We focused our *in vivo* work on CXCL1 in mice and gerbils because it is the rodent equivalent of human CXCL8, a chemoattractant for neutrophils and T cells[35]. It is established that expression of *CXCL8* mRNA and production of CXCL8 is enhanced in gastric tissues of *H. pylori*-infected versus uninfected individuals[36]. In *H. pylori*-positive patients, serum CXCL8 levels were further elevated in those with gastric cancer[37], and CXCL8 has been implicated in the development, severity, and spread of gastric carcinoma[38, 39]. In the present report, CXCL1 was associated with dysplasia/carcinoma in mice and gerbils infected with *H. pylori*, and gefitinib treatment reduced both parameters. Similarly, studies in hepatocellular carcinoma and in non-small cell lung cancer cell lines have demonstrated that EGFR activation leads to increased expression of CXCL8, and this supports the tumor microenvironment[40, 41]. Patients with hepatocellular carcinoma have increased levels of serum CXCL8, and this correlates with larger tumor size, more advanced stage and worse survival[42]. In a mouse model of carcinogen-induced lung tumorigenesis, gefitinib reduced tumor multiplicity and burden, and this was associated with decreased expression of pro-inflammatory genes in a microarray analysis[13].

Our results indicate that EGFR signaling in epithelial cells is critical for inflammation after *H. pylori* infection. GECs isolated from infected mice and gerbils treated with gefitinib and mice with epithelial-specific deletion of *Egfr* express less chemokines and cytokines, associated with decreased MPO-positive cells and DNA damage. It should be noted that myeloid cells are also a source of CXCL8 in *H. pylori* infection[43]. Because we reported that myeloid-specific knockout of EGFR also leads to a diminution of chemokine and cytokine expression in the gastric tissues of *H. pylori*-infected mice[17], gefitinib may also reduce EGFR signaling in gastric macrophages, thus amplifying the protective effect of EGFR inhibition in gastric carcinogenesis.

Eradication therapy for *H. pylori* has been proposed as a strategy to reduce gastric cancer incidence [2]. However, this approach may not be effective in patients with premalignant lesions. Our data indicate that pEGFR is still abundant in mice as well in humans after *H. pylori* has been eradicated. Thus, persistent activation of EGFR even after the bacterium has been eliminated can play an important role in the carcinogenic process. Indeed, *H. pylori* eradication in INS-GAS mice did not completely prevent development of carcinoma. However, double treatment with antibiotics plus gefitinib completely prevented development of intramucosal carcinoma.

The p38/NF- κ B pathway[44, 45] and/or MAPK1/3 signals[46, 47] have been linked to *H. pylori*-induced *CXCL8* transcription. It has been suggested that both AP-1 and NF- κ B are required for maximal induction of *CXCL8* mRNA expression[48]. In our report, inhibitors of EGFR, MAPK1/3, or AP-1 decreased *CXCL8* mRNA expression in infected human AGS cells. Further, gefitinib also blocked MAPK1/3 and AP-1 activation, and MAPK1/3 inhibition decreased JUN nuclear translocation, demonstrating that EGFR signals through a MAPK1/3-AP-1 transduction pathway to stimulate *CXCL8* gene expression.

Supporting these data, it has been demonstrated that the Type IV Secretion System pilus component, CagL, activates EGFR and ERBB3, as well as focal adhesion kinase and SRC, by mimicking the function of fibronectin [49]. Additionally, CagL can lead to ADAM-17-mediated release of EGF, resulting in MAPK1/3 activation[50]. In addition, induction of human β -defensin-3 or matrix metalloproteinase-10 expression in *H. pylori*-infected GECs requires an EGFR/MAPK1/3 transduction pathway[28, 51]. Notably, chronic *in vitro* infection with *H. pylori* for 24 h has been reported to suppress the ability of EGF ligand to activate EGFR [28]. However, we have shown herein that experimental *H. pylori* infection *in vivo* leads to increased pEGFR beginning at 1 day post-infection and continuing out to chronic time points (4 weeks and 8 weeks), and that humans exhibit epithelial pEGFR staining after many years of infection that can persist even after *H. pylori* eradication. Growth factors like EGF are capable of triggering NF- κ B activation in breast, prostate, and lung cancer cell lines through EGFR phosphorylation[52]. However, we did not observe an effect of gefitinib on NF- κ B activation. This is not surprising, considering that *H. pylori* possesses a variety of virulence factors that can activate NF- κ B through multiple cellular receptors, including Toll-like receptors and NOD1[53, 54]. Importantly, we recapitulated the concordance between EGFR and MAPK activation *in vivo*: C57BL/6 and INS-GAS mice infected with *H. pylori* had concomitantly increased levels of pEGFR and pMAPK1/3, which were inhibited with gefitinib treatment.

We also showed that gefitinib not only dampens the innate immune response of GECs and consequently their ability to recruit PMNs, but also inhibits *H. pylori*-induced DNA damage in GECs. This effect further supports the potential benefits of gefitinib. Our studies provide new insight into the mechanism by which *H. pylori* mediates gastric carcinogenesis, and we propose that gefitinib could represent an alternative therapeutic approach to limit gastric cancer development by decreasing inflammation and by inhibiting DNA damage in GECs. Related to this concept, *H. pylori* strains isolated from patients in regions with low risk of developing gastric cancer induce less CXCL8 and DNA damage than those from high-risk regions[21, 55, 56]. In addition, the interaction of the host and *H. pylori* genetics, according to their phylogeographic variations, is a critical feature for the development of gastric diseases, such that loss of co-evolution increases cancer risk[55, 57]. Therefore, determination of EGFR activation in individuals with different genetic origins and/or infected with *H. pylori* of varying phylogeographic origin is a future direction for our studies. It could prove important to consider human and bacterial genetic factors and specific host signaling responses in developing personalized chemoprevention strategies, such as utilization of EGFR inhibition in populations at high risk for *H. pylori*-induced gastric cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Whole slide imaging and quantification of immunostaining were performed in the Digital Histology Shared Resource at Vanderbilt University Medical Center (www.mc.vanderbilt.edu/dhsr). Immunofluorescence confocal imaging was performed in the Vanderbilt Cell Imaging Shared Resource.

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Significance of this study

What is already known on this subject?

- *Helicobacter pylori* infection is the primary cause of gastric cancer, which remains a major public health problem as the third leading cause of cancer deaths.
- Antibiotic-based eradication of *H. pylori* may not be of benefit once advanced histologic lesions have occurred, indicating that other chemopreventive strategies are needed.
- Epidermal growth factor receptor (EGFR) overexpression and transactivation (phosphorylation) is associated with gastric carcinogenesis in humans, but EGFR has not been investigated in animal models of *H. pylori*-induced gastric dysplasia and carcinoma.

What are the new findings?

- Administration of a clinically available EGFR inhibitor, gefitinib, blocked *H. pylori*-stimulated phosphorylation of EGFR in gastric epithelial cells in animals, and in vitro.
- Gefitinib administration reduced infiltration of inflammatory cells, chemokine and cytokine expression, DNA damage, and development of gastric dysplasia and carcinoma in mouse and gerbil models of *H. pylori*-induced gastric cancer. C57BL/6 mice with genetically-engineered epithelial-specific knockout of *Egfr* also exhibited attenuated inflammation and DNA damage.
- Epithelial EGFR signaling occurred through MAPK1/3 (ERK1/2) and AP-1 pathways, which thus may contribute to the pro-inflammatory and pro-tumorigenic responses.
- Antibiotic eradication of *H. pylori* did not reduce EGFR phosphorylation in a human longitudinal cohort or in INS-GAS mice, and gefitinib significantly reduced gastric carcinoma in mice treated with antibiotics.

How might it impact on clinical practice in the foreseeable future?

- These studies provide evidence that EGFR inhibition may be an effective strategy for the chemoprevention of *H. pylori*-associated gastric carcinogenesis, particularly in individuals at high-risk for neoplastic progression.

Figure 1.

Levels of pEGFR and pH2AFX in C57BL/6 mice infected with *H. pylori* PMSS1 on control or gefitinib-supplemented diet. (A) Western blot for pEGFR (Y1068) and total EGFR in GECs isolated 7 days post-infection. (B) Immunohistochemistry for pEGFR in gastric tissues after 4 weeks infection. (C) Percentage of cells pH2AFX-positive by flow cytometry analysis. (D and E) Quantification of nuclei positive for pH2AFX, and representative pH2AFX immunohistochemistry. * P <0.05, *** P <0.001 versus uninfected mice; § P <0.05, §§ P <0.01 versus PMSS1-infected on control diet. In B and E, scale bar = 100 μ m.

Figure 2.

MPO and chemokine/cytokine levels in C57BL/6 mice infected with PMSS1 for 4 weeks and maintained on control or gefitinib-supplemented diet. (A) Quantification of MPO-positive cells assessed by immunohistochemistry. (B) Representative MPO staining in gastric tissues. Scale bar = 100 μ m in upper panel. Scale bar = 50 μ m in lower panel. (C) Chemokine and cytokine levels in gastric tissue lysates assessed by Luminex assay. * P <0.05, *** P <0.001 versus uninfected mice; § P <0.05, §§ P <0.01, §§§ P <0.001 versus PMSS1-infected on control diet.

Figure 3.

Effect of *Egfr* deletion on pEGFR, MPO, pH2AFX, and chemokine expression with *H. pylori* PMSS1 infection for 4 weeks. (A) Western blot for EGFR in GECs isolated from naive *Egfr^{fl/fl}* and *Egfr^{Δepi}* mice. (B) Immunohistochemistry for pEGFR in gastric tissues of infected *Egfr^{fl/fl}* and *Egfr^{Δepi}* mice. (C) Quantification of MPO-positive cells assessed by immunohistochemistry. Scale bar = 100 μ m. (D) *Cxcl1* (Upper panel) and *Cxcl2* (Lower panel) mRNA expression assessed by real-time PCR in isolated GECs. (E) Number of

pH2AFX-positive nuclei by immunohistochemistry. * $P<0.05$, ** $P<0.01$ versus uninfected mice; § $P<0.05$, §§ $P<0.01$ versus PMSS1-infected on control diet.

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Figure 4.

FVB/N INS-GAS mice infected for 8 weeks with PMSS1 and maintained on control or gefitinib-supplemented diet. (A) Frequency of diagnoses in INS-GAS mice. The adjacent numbers correspond to the number of mice in each section of the bar. ¶¶¶ $P=0.0003$ for PMSS1-infected on gefitinib versus control diet, comparing intramucosal carcinoma frequency; # $P=0.044$, comparing frequency of carcinoma + low-grade dysplasia (red + blue bars). LGD; low-grade dysplasia. (B) Extent of dysplasia and carcinoma quantified as a percentage of the total area of the gastric mucosa in H&E-stained tissues. (C) H&E-stained

stomach tissues, showing intramucosal carcinoma (indicated by the circle) on the control diet, versus low-grade dysplasia with gefitinib. (D) Quantification of gastric tissue MPO-positive cells by immunohistochemistry. (E) *Cxcl1* and *Cxcl2* mRNA expression by real-time PCR on isolated GECs. (F) CXCL1 protein levels quantified by ELISA from GEC lysates. (G) Number of pH2AFX-positive nuclei in gastric tissues. In B-G, * $P < 0.05$, ** $P < 0.01$ versus uninfected mice; § $P < 0.05$, §§ $P < 0.01$ versus PMSS1-infected on control diet.

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Figure 5.

Mongolian gerbils infected with *H. pylori* strain 7.13 for 8 or 12 weeks and maintained on control or gefitinib-supplemented diet. (A) H&E staining, showing invasive adenocarcinoma with control diet and low-grade dysplasia with gefitinib diet. Scale bar = 100 μ m. (B) Frequency of invasive adenocarcinoma. (C) Quantification of MPO-positive cells assessed by immunohistochemistry in gastric tissues. (D) *Cxcl1* and *Il1b* mRNA expression in gastric tissues by real-time PCR. (E and F) pH2AFX quantification, number of positive nuclei per high power field (HPF), and representative staining in gastric tissues. Scale bar = 100 μ m in

upper panel. Scale bar = 50 μm in lower panel. In B-F, gerbils were infected for 12 weeks. Scale bar = 100 μm . ** $P<0.01$, *** $P<0.001$ versus uninfected mice; § $P<0.05$, §§ $P<0.01$ versus 7.13-infected on control diet.

Figure 6.

Effect of gefitinib on chemokine and pH2AFX levels in mouse gastric epithelial cells (ImSt). (A) *Cxcl1* and (B) *Cxcl2* mRNA expression by real-time PCR in cells co-cultured with *H. pylori* strains PMSS1 or 7.13 for 6 h. (C) CXCL1 levels quantified by ELISA in supernatants of ImSt cells co-cultured with *H. pylori* for 24 h. (D) Percentage of pH2AFX positive cells by flow cytometry after infection for 24 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus uninfected cells; § $P < 0.05$, §§ $P < 0.01$ versus *H. pylori*-infected cells.

Figure 7.

Effect of EGFR inhibition on chemokine production and MAPK/AP-1 pathway activation.

(A) *CXCL8* mRNA expression in AGS cells pre-treated with gefitinib (G), the MEK inhibitor PD 98059 (P) or the AP-1 inhibitor SR 11302 (S) and infected with *H. pylori* strains PMSS1 or 7.13 for 2 h. *** $P < 0.001$ versus uninfected cells; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ versus *H. pylori*-infected cells. (B) Western blot for JUN and fibrillar (FBL) in nuclear lysates of AGS cells pre-treated with inhibitors and infected with *H. pylori* for 1 h. (C) Western blot for pMAPK1/3 and pRELA in AGS cells pre-treated with gefitinib and infected with *H. pylori* for 1 h. (D) Immunohistochemistry for pMAPK1/3 and pJUN in gastric tissues from C57BL/6 mice infected for 4 weeks. (E) Immunohistochemistry for

pMAPK1/3, pJUN, and pEGFR in gastric tissues from INS-GAS mice infected for 8 weeks. Scale bar = 100 μm in pMAPK1/3 and pEGFR photomicrographs. Scale bar = 50 μm in pJUN photomicrographs.

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Figure 8.

Effect of *Egfr* deletion on pEGFR, pMAPK1/3, pJUN, and chemokine expression induced by *H. pylori* in GEC monolayers. (A) pEGFR, pMAPK1/3, and pJUN immunofluorescent staining of GEC monolayers isolated from *Egfr^{fl/fl}* and *Egfr^{epi}* mice, and infected with PMSS1 for 1 h at MOI 10. pEGFR, pMAPK1/3 and pJUN in green, actin in red, and nuclei in blue, imaged by confocal microscopy. Scale bar = 20 μ m. (B) *Cxcl1* and *Cxcl2* mRNA levels by real-time PCR in gastric glands isolated from *Egfr^{fl/fl}* and *Egfr^{epi}* mice, and infected for 24 h with PMSS1. ** $P < 0.01$, *** $P < 0.001$ versus uninfected cells; \$\$ $P < 0.01$, \$\$\$ $P < 0.001$ versus *H. pylori*-infected cells.

Figure 9.

(A) Effect of *H. pylori* eradication on pEGFR in human gastric biopsies. All individuals were confirmed to be *H. pylori*-negative on follow-up. (B) Immunohistochemistry for pEGFR in pre- and post-eradication biopsies. (C) Frequency of diagnoses in INS-GAS mice. The number of mice in each section of the bar is noted. $\nabla P < 0.05$, $\nabla\nabla P < 0.01$, for carcinoma versus PMSS1 only; $\# P < 0.05$, for carcinoma versus PMSS1 + antibiotics (ABX). When dysplasia + carcinoma is considered, for gefitinib + ABX, $P < 0.05$ versus ABX only, and $P < 0.01$ versus PMSS1 only. (D) Representative H&E staining of gastric tissues from

infected mice in the different treatment groups. PMSS1 only: intramucosal carcinoma; gefitinib or ABX: dysplastic glands; gefitinib + ABX: non-dysplastic tissue. (E) Quantification of pEGFR staining. * $P < 0.05$ versus PMSS1 only. (F) Representative pEGFR immunohistochemistry. Scale bar = 100 μm .

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