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Original article

In-Cell Western analysis of *Helicobacter pylori*-induced phosphorylation of extracellular-signal related kinase via the transactivation of the epidermal growth factor receptor

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Abstract

Helicobacter pylori activates extracellular-signal related (ERK) kinases in gastric epithelial cells, via transactivation of the EGF receptor (EGFR). *H. pylori* activation of EGFR may be relevant to epithelial hyperproliferation and gastric carcinogenesis. The aim of this study was to develop an 'In-Cell Western' (ICW) assay for quantitative examination of *H. pylori*-induced epithelial signalling, to enable the role of the EGFR in *H. pylori*-induced phosphorylation of ERK in epithelial cells to be ascertained. *H. pylori* strains were co-incubated with A431 and AGS cells. pERK and total ERK were quantified *in situ* using ICW analysis. *H. pylori* strains both with, and without a *cag* PAI, and *Helicobacter felis*, significantly increased pERK levels in A431 cells. The EGFR inhibitor EKB-569 dose-dependently reduced *H. pylori*-induced ERK phosphorylation in A431 and AGS cells. A significantly lower reduction was observed with *cag*+ strains in A431 but not AGS cells. The *cag* PAI was not necessary for EGFR signal transactivation. These data suggest that *H. pylori* induces pERK in epithelial cells partly via the EGFR pathway. Additional signalling mechanisms are likely to be involved in *H. pylori*-induced ERK phosphorylation. ICW analysis is a rapid quantitative method for evaluating the effects of inhibitors on *H. pylori*-induced cell signalling pathways of relevance to gastric carcinogenesis.
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Keywords: *Helicobacter pylori*; EGFR; ERK; Phosphorylation; EKB-569; EGFR inhibitor; 'In-Cell Western' assay

1. Introduction

Infection with *Helicobacter pylori* increases the risk of developing distal gastric cancer [1–5]. Both the long-term inflammatory response induced by *H. pylori* [1,2], and bacterial-induced epithelial cell signalling pathways are likely to be contributory factors promoting increased risk of neoplasia [3–5]. *Helicobacter* infection in humans [6,7], and animal models [8–11], is associated with marked hyperproliferation of gastric epithelial cells. Recent *in vitro* evidence from bacterial-epithelial co-culture systems has identified that *H. pylori* activates several signalling pathways of relevance to epithelial hyperproliferative responses [3–5]. *H. pylori* interacts with host target molecules such as the epidermal growth factor receptor (EGFR) [12,13], Her2/Neu [14] and hepatocyte growth factor receptor c-Met [14]. Furthermore, epithelial translocation of CagA, via the *cag* pathogenicity island (*cag*PAI) deregulates epithelial signalling in both a tyrosine phosphorylation-dependent and -independent manner [5].

Several studies using Western blotting have demonstrated that *H. pylori*, and bacterial culture supernatant, activate

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular-signal related kinase; ICW, 'In-Cell Western'; MAPK, mitogen-activated protein kinase; PAI, pathogenicity island.

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extracellular-signal related kinase 1/2 (ERK1/2) in gastric epithelial cells in a *cag*PAI-independent manner [15,16]. Activation of the ERK pathway by *H. pylori* is considered, in part, to be via tyrosine kinase receptors such as EGFR [12,13]. Both *cag*PAI- independent [12] and dependent [13] epithelial EGFR transactivation by *H. pylori* have been reported. The activation of the mitogen-activated protein kinase (MAPK)-ERK kinase pathway in epithelial cells by *H. pylori* is required for up-regulation of heparin binding-epidermal growth factor [12], the mediator of *H. pylori*-stimulated EGFR transactivation [12]. The translocated CagA protein of *H. pylori* and subsequent CagA mediated activation of protein tyrosine phosphatase SHP-2 [17], also induces sustained ERK activation in epithelial cells in a Ras-independent manner [18]. Hyperactivation of the MAPK-ERK pathway induced by *H. pylori* in epithelial cells is likely to result in dysregulated epithelial proliferative responses [3,5]. Variations in the ability of *H. pylori* strains to activate ERK may be relevant to the role of *H. pylori* in promoting atrophic gastritis and gastric cancer.

'In-Cell Western' assay allows quantitative and highly sensitive cell-based analysis of phosphorylation of receptor tyrosine kinases and intracellular signalling proteins such as ERK [19,20]. In addition, the technology allows rapid, quantitative, high throughput analysis of kinase inhibitors on signalling pathways [19]. To date, the technology has largely been applied to evaluate cellular signalling responses following ligand-induced activation of cell surface receptors. The potential of the technology to evaluate pathogen-induced cell signalling responses has not been evaluated. The aim of the present study was to develop an ICW assay for rapid, quantitative analysis of *H. pylori*-induced epithelial signalling responses. The established ICW assay for *H. pylori*-induced activation of ERK in epithelial cells was used to evaluate the contribution of pathogen-induced EGFR transactivation in ERK phosphorylation.

2. Material and methods

2.1. Cell culture

A431 cell (human squamous carcinoma), and four human gastric epithelial cell lines (AGS, MKN-1, MKN-28 and ST42), were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 2 mM L-glutamine at 37 °C in 5% CO_2. Cells were seeded into 96-well plates (Nunc, Rochester, NY, USA) at a density of $3-5 \times 10^4$ cells per well and cultured until 80–90% confluent. Cells were cultured in serum free medium 24 h prior to assay.

2.2. EGF responses of epithelial cells

A431 cells and gastric cell lines were incubated with recombinant human EGF (Upstate, NY, USA) (1–200 ng/ml) in serum free RPMI for 10 min. The optimized EGF concentration for total tyrosine, EGFR, and ERK1/2 phosphorylation status using the ICW assay was determined, and used as a positive control in subsequent studies.

2.3. Bacterial-epithelial co-culture

H. pylori strains NCTC11637 and G27, which contain the *cag*PAI, G27Δ*cag*M mutant (kindly provided by Dr A. Covacci), SS1 which has a non-functional *cag*PAI [21], G50 (which lacks a *cag*PAI), *H. pylori* strains (16GX, 42GX, 47GX) and *H. felis* were used. Strains were grown on a 7% (v/v) horse blood agar plate under microaerobic conditions at 37 °C for 2 days. Bacteria were harvested into serum and antibiotic-free RPMI 1640 medium and re-suspended at 5×10^7/ml. Bacteria were co-cultured with epithelial cells for 45 min to 3 h. Untreated A431 and AGS cells, and cells cultured with EGF (25 ng/ml) for 10 min were used as negative and positive control, respectively. In some experiments cells were cultured with purified *H. pylori* lipopolysaccharide (LPS) (kindly provided by Dr A. Moran).

2.4. Inhibition of EGF and H. pylori-induced ERK phosphorylation

A431 and AGS cells were pre-incubated EKB-569 (kindly provided by Dr L. Greenberger) for 1 h at 37 °C before stimulation with EGF for 10 min. A431 and AGS cells similarly pretreated with EKB-569 were incubated with *H. pylori* for 90 min (AGSA) or 3 h (A431). Cells pre-incubated with EKB-569 and cultured without bacteria were used as negative controls. Cell viability in experiments was determined by Trypan blue exclusion.

2.5. 'In-Cell Western' assay

The medium was aspirated and the cells fixed with 3.7% (v/v) formaldehyde (VWR, Poole, UK) in phosphate buffered saline (PBS). Cells were permeabilized with 0.1% (v/v) Triton X-100 (VWR, Poole, UK) in PBS, and non-specific protein binding sites blocked with 100 µl/well Odyssey blocking buffer (Licor Bioscience, Lincoln, NE, USA).

All antibody pairs used were diluted in Odyssey blocking buffer. For the detection of ERK phosphorylation, mouse-monoclonal anti-pERK1/2 (Santa Cruz, CA, USA) and rabbit-polyclonal anti-ERK1 (Santa Cruz) antibodies were used. Mouse-monoclonal anti-phosphotyrosine antibody (Upstate, Lake Placid, NY, USA) was combined with rabbit anti-EGFR antibody (Cell Signaling, Beverly, MA, USA) to detect the total tyrosine phosphorylation (PY) following EGF or bacterial-stimulation. For EGFR phosphorylation, mouse-monoclonal anti-phospho-EGFR (Tyr1173) (Upstate) was combined with rabbit anti-EGFR antibody (Cell Signaling). After incubation, plates were washed with 0.1% Tween-20 (v/v) in PBS before incubation with secondary antibodies, goat anti-mouse IgG Alexa Fluor 680 (Molecular Probes, Eugene, OR, USA) to detect PY, pEGFR and pERK signals or goat anti-rabbit IgG IRDye 800CW (Rockland, Gilbertsville, PA, USA) for detecting total ERK1 and EGFR levels, respectively. Initially, phosphorylated antibodies and pan protein antibodies were analysed both separately, and in combination, to check for potential interference. All antibodies used were tested for non-specific binding with

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