Helicobacter pylori Infection Induces Phosphorylation of ATM/ATR Substrates in Host Cells

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Abstract: In this study we report for the first time that infection with wild-type (cag PAI-positive) and corresponding isogenic cag PAI negative mutant induces activation of wide variety of substrates in both cell lines. Then we show activation of Chk2 after infection with H. pylori P12, also we detected p-Chk2 after long-time infection. Using ATM/ATR inhibitor we found that Chk2 was activated by kinase different from ATM/ATR in HeLa and AGS cells. At the same time, we do not detect activation of p53, one of the substrates of Chk2. We conclude, that infection of eukaryotic cells with H. pylori leads to activation ATM/ATR substrates, that confirms the damage of DNA induced by this microorganism.

Key words: Helicobacter pylori • DNA damage • Chk2, ATM/ATR • Kinase • Phosphorylation

INTRODUCTION

Helicobacter pylori induces an inflammatory response in the stomach that persists for decades and increases the risk not only for peptic ulceration, but also for gastric adenocarcinoma and non-Hodgkins lymphoma of the stomach [1,2]. Gastric adenocarcinoma is the second leading cause of cancer-related death in the world and chronic gastritis induced by H. pylori is the strongest known risk factor for this malignancy [1,3-7]. Little is known about the influence of Helicobacter pylori infection on genetic material of the host cell. At the core of the DNA damage signaling apparatus are a pair of related protein kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), which are activated by DNA damage [8]. ATM/ATR kinases phosphorylate different downstream targets (Chkl, Chk2, p53, BRCA1 etc.) that initiate cell cycle arrest, DNA repair, or apoptosis [9-11].

Previous studies connecting H. pylori infection with DNA damage of epithelial cells were carried out using tissue samples of mouse stomach [12] and human stomach [13].

In this study we show that infection of eukaryotic cells with H. pylori leads to activation ATM/ATR substrates, that confirms the damage of DNA induced by this microorganism.

MATERIALS AND METHODS

Helicobacter pylori Strains: H. pylori P12 is a clinical isolate obtained from a patient with duodenal ulcer (Hamburg, Germany) [14]. H. pylori ΔPAI is a mutant from P12 lacking the PAI [15]. For cultivation, the bacteria were resuspended in brain heart infusion (BHI, Difco, Detroit, USA) medium containing vancomycin (10 µg/ml), trimetoprim (1,25 µg/ml) and nisantin (1 µg/ml) for H. pylori P12 and vancomycin (10 µg/ml), trimetoprim (1,25 µg/ml), nisantin (1 µg/ml) and kanamycin (8 µg/ml) for H. pylori ΔPAI at 37°C in a microaerophilic atmosphere (generated by Campy Gen, Oxoid, Basingstoke, England).

Cell Culture and H. pylori Infection: AGS cells (ATCC CRL 1739, a human gastric adenocarcinoma epithelial cell line) were cultivated in 6-well tissue culture dishes using RPMI 1640 medium at 37°C in a humidified atmosphere of 95% air and 5% CO2 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) for 2 days to reach monolayers of about 70% confluence. 24h before infection, the medium was replaced by fresh RPMI 1640 medium. For the infection, the bacteria were harvested in PBS (pH 7.4) using sterile tubes, washed with PBS, diluted corresponding to the multiplicity of infection (MOI) as described in the figure. Cells were incubated for different periods of time in the absence (controls) or
Fig. 1: Activation of ATM/ATR substrates in AGS cells after infection with *H. pylori* P12 wild-type (cag PAI-positive) (a) and *H. pylori* ? PAI corresponding isogenic cag PAI negative mutant (b) at MOI of 100. k - control cells, 1-4 - corresponding infection for 30min, 90min, 3h and 6h.

Fig. 2: Activation of Chk2 kinase in AGS (a) and HeLa (b) cells after infection with *H. pylori* P12 at MOI of 100. k - control cells; 1-3 - corresponding infection for 30min, 90min, 3h; * - treatment with ATM/ATR inhibitor; d - treatment with doxorubicin.

Fig. 3: Activation of Chk2 kinase in AGS (a) and HeLa (b) cells after infection with *H. pylori* P12 at MOI of 100. k - control cells; 1-2 - corresponding infection for 6h and 24 h.

Fig. 4: Activation of p53 protein in HeLa cells after infection with *H. pylori* P12 at MOI of 100. k - control cells; 1-3 - corresponding infection for 30min, 90min, 3h; * - treatment with ATM/ATR inhibitor; d - treatment with doxorubicin.
presence of the bacteria. Infection with *H. pylori* was routinely monitored by light microscopy.

**Western Blotting:** *H. pylori*-infected and uninfected cell lines were lysed in buffer containing (%): SDS-2, β-mercaptoethanol-3, glycerin-20, bromphenol blue-0.05 and 5 mM dithiotrehit. Proteins (30 Mg) were separated by SDS-PAGE in 10% polyacrylamide gels [16] and transferred to polyvinylidene difluoride (PVDF) membranes (Poly Screen NEF 1002). Membranes were blocked in TBS-T (140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, 1% (V/V) Tween_20) with 3% bovine serum albumin for 1h at room temperature and washed three times with TBS-T. Phospho-substrate levels were examined in AGS and HeLa cells by Western blotting using Phospho-(Ser/Thr) ATM/ATR Substrate Antibody and anti-p-p53 antibodies (Cell Signaling Technology). Phosphorylation form of Chk2 was detected with anti-p-Chk2 antibody (Cell Signaling Technology) and the same blot was used for anti-β-actin immunoblotting. Between blotting procedures, the membranes were stripped for 30 min at 50°C in stripping buffer (62.5 mM Tris-HCl pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS).

Primary antibodies were detected using horseradish peroxidase conjugated secondary antibodies (HRP α-mouse and HRP α-rabbit) (Amersham) and visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) according to the manufacturer’s instructions.

**Treatment with Doxorubicin and ATM/ATR Inhibitor:** ATM kinase was activated by treatment with doxorubicin (1 μM) (Sigma) for 4h in RPMI medium [17]. ATM/ATR inhibitor (Calbiochem) was used at a final concentration of 2.5 μg/ml for HeLa and 0.7 μg/ml for AGS [18]. Cells were pretreated for 18h before infection.

**RESULTS AND DISCUSSION**

To investigate activation of any ATM/ATR substrates, we screened for phosphorylation potential ATM/ATR substrates using phospo-specific antibodies against known ATM/ATR substrates (Fig. 1). We identified proteins cross-reacting to phospo-specific antibodies in response to *H. pylori* infection by Western-blot analyze.

These results show that infection at MOI of 100 (bacteria pro cell) leads to phosphorylation of several substrates with molecular masses (mm) 62kDa and 56 kDa after 30 min of incubation.

We report that infection with wild-type (cag PAI-positive) and corresponding isogenic cag PAI negative mutant induces activation of wide variety of substrates in AGS cells.

Then we show activation of Chk2 after infection with *H. pylori* P12 for different time, using antibody against phosphorylated form of this kinase (Fig. 2). Both cell lines were infected at MOI of 100, after 30min we detected p-Chk2 and observed the increasing level of phosphorylation after during the time of infection. Using ATM/ATR inhibitor we found that Chk2 was activated by kinase different from ATM/ATR in HeLa and AGS cells.

Also we detected p-Chk2 after long-time infection (24h) (Fig. 3). In non-infected cells the low level of Chk2 phosphorylation after incubation in RPMI medium without FCS for 24h was also registered.

At the same time, we do not detect activation of p53, one of the substrates of Chk2 (Fig. 4).

**CONCLUSION**

We conclude, that infection of eukaryotic cells with *H. pylori* leads to activation ATM/ATR substrates, that confirms the damage of DNA induced by this microorganism. Thus, our data contribute to evidence of ATM/ATR activation mediated through damage of host DNA during *H. pylori* infection.

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**REFERENCES**