Suppression of CD74 Expression and *Helicobacter pylori* Adhesion by Auraptene Targeting Serum Starvation-Activated ERK1/2 in NCI-N87 Gastric Carcinoma Cells

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Received December 9, 2009; Accepted January 19, 2010; Online Publication, May 7, 2010

**Key words:** *Helicobacter pylori*; CD74; adhesion; IL-8; auraptene

*Helicobacter pylori* (*H. pylori*) is a major human pathogen and plays a central role in chronic gastritis and gastric cancer. Since the adhesion of *H. pylori* to the human gastric epithelium is the initial and critical step of its infection, anti-*H. pylori* adhesion agents may be effective for the prevention and therapy of *H. pylori*-associated diseases. CD74 has recently been identified as a new receptor for *H. pylori* urease, and we have previously reported that several citrus components strongly suppressed CD74 expression in NCI-N87 gastric carcinoma cells. We found in this present study that auraptene (citrus coumarin) disrupted serum starvation-induced extracellular signaling-regulated kinase (ERK) 1/2 activation and attenuated *H. pylori* adhesion and IL-8 production in a co-culture system. In addition, the knockdown of CD74 expression led to a significant decrease of *H. pylori* adhesion, but unexpectedly increased IL-8 production. However, PD98059 (a MEK1/2 inhibitor) dramatically down-regulated this cytokine, suggesting MEK/ERK-dependent IL-8 production. Our results suggest that auraptene suppressed *H. pylori* adhesion and resulting chemokine production by disrupting ERK1/2 activation.

**Abbreviations:** AUR, auraptene; CagA, cytotoxin-associated antigen A; EGFR, epidermal growth factor receptor; ERK, extracellular signaling-regulated kinase; MAPK, mitogen-activated protein kinase; MIF, macrophage migration inhibitory factor; NF-κB, nuclear factor-kappa B

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**Received December 9, 2009; Accepted January 19, 2010; Online Publication, May 7, 2010**

**[doi:10.1271/bbb.90910]**

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Auraptene Suppresses CD74 and H. pylori Adhesion

Hand, we have previously reported that an auraptene treatment attenuated inflammatory leukocyte activation in vivo and led to decreased inflammation, H₂O₂ production, and cell proliferation, while also suppressing 12-O-tetradecanoylphorbol-13-acetate-induced superoxide in HL-60 cells.²⁰ These findings suggest that auraptene would mitigate oxidative stress by suppressing the generation of oxygen radicals by inflammatory leukocytes.

We have previously reported that auraptene notably suppressed CD74 expression by an unknown mechanism.²¹ Interestingly, Takeda et al. have indicated that an oral administration of auraptene inhibited H. pylori colonization in Mongolian gerbils.²² However, this study did not clarify its mechanisms of action. We thus attempted in this present study to elucidate the molecular mechanism by which auraptene suppresses CD74 in NCI-N87 human gastric carcinoma cells. The effects of auraptene on H. pylori adhesion and the resulting cytokine production were also investigated.

Materials and Methods

Chemicals. RPMI1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (NY, USA), and oligonucleotide primers were synthesized by Sigma Genosys (Hokkaido, Japan). Antibodies were purchased from the following sources: rabbit anti-CD74, Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-phospho-extracellular regulated kinase (ERK) 1/2, Cell Signaling Technology (MA, USA); mouse anti-u-tubulin, Calbiochem (San Diego, CA, USA); rabbit anti-H. pylori, anti-rabbit IgG, anti-mouse IgG and nonspecific IgG, Dako (Glostrup, Denmark). siRNA was purchased from Wako Pure Chemicals (Osaka, Japan), and auraptene was isolated as previously described.²⁰,²³ All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless specified otherwise.

Gastric cell culture. NCI-N87 gastric carcinoma cells were obtained from American Type Culture Collection (Rockville, MD, USA), and grown in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Bacterial culture. H. pylori NCTC 11637 was purchased from American Type Culture Collection, and grown on Columbia HP agar plates (Beckton Dickinson, CA, USA) at 37°C under microaerophilic conditions. The bacteria were transferred after 72 h into Brucella plates (Beckton Dickinson, CA, USA) at 37°C, 100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Reverse transcription-polymerase chain reaction. The cells (1.0 × 10⁶ cells/5.0 ml) were seeded onto a 60-mm dish and preincubated for 24 h. The medium was replaced with serum-free RPMI1640 containing the samples dissolved in DMSO. After incubating for the designated times, the cells were lysed with a lysis buffer (1% protease and phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA), 10 mm Tris (pH 7.4), 1% sodium dodecyl sulfate (SDS), and 1 mM sodium orthovanadate) and the lysate was sonicated. The denatured proteins (30 μg) were separated by using SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gel (the non-specific control and CD74; 45 pmol/0.6 ml) by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions.

Knockdown of CD74. siRNA was transfected with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. Briefly, 24 h before transfection, the cells (2.0 × 10⁶ cells/1.0 ml) were seeded onto a 24-well microplate in RPMI1640 with 10% FBS. The cells (about 70% confluent) were transfected with siRNA (the non-specific control and CD74; 45 pmol/0.6 ml) by using Lipofectamine™ 2000 dissolved in Opti-MEM I™ for 7 h. The cells were then incubated for 48 h after the medium had been replaced by 1 ml of RPMI1640 containing 10% FBS. After being washed twice with PBS, the cells were treated with serum-free RPMI1640 containing H. pylori (2.0 × 10⁶ cells/1.0 ml). H. pylori adhesion and the quantification of IL-8 were respectively evaluated after 6 h and 24 h as already described.

Cell viability. Cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT).²⁶ After being incubated, the cells were washed twice with PBS, then 110 μl of serum-free RPMI1640 containing 10 μl of an MTT solution (5 mg/ml)
was added to each culture, and the culture was further incubated at 37 °C for 2 h. Next, 200 μl of DMSO was added, the culture was sonicated for 5 min, and then 100 μl of HCl/2-propanol (3.4 μl/ml) was added to each well. The visible absorbance was measured at 570 nm and 630 nm with a microplate reader. The cell viability in each experiment was >70%. Statistical analysis. Each experiment was performed at least three times and the data are shown as the mean ± standard deviation (SD), where appropriate. A one-way analysis of variance (ANOVA) was performed, followed by Tukey’s multiple-comparison test to determine whether the differences among the groups were significant. The differences are considered significant at p < 0.05 or 0.01.

Results

Suppressive effects of auraptene on ERK1/2 activation

As shown in Fig. 1B, serum starvation for 1 h resulted in marked dephosphorylation of ERK1/2, after which it was time-dependently restored during 3–12 h. It is notable that auraptene significantly attenuated ERK1/2 activation after 6 h (p38 MAPK and JNK 1/2, data not shown).

Auraptene inhibited H. pylori adhesion and resulting IL-8 production

We wanted to find if auraptene could suppress H. pylori adhesion and the resulting production of pro-inflammatory cytokines in a co-culture system. The level of H. pylori adhesion to NCI-N87 cells was slightly but significantly decreased by auraptene at a concentration of 50 μM (Fig. 2A). We then assessed whether the auraptene treatment could affect the production of IL-8 and MIF in NCI-N87 cells exposed to H. pylori. Exposure to H. pylori for 24 h increased the level of IL-8 production by 2.1-fold. Interestingly, pretreating with auraptene for 48 h significantly inhibited the production in concentration-dependent fashion, the level being reversed to the control value (Fig. 2B). The MIF level was not affected with any statistical significance (Fig. 2C).

Knockdown of CD74 inhibited H. pylori adhesion

To ascertain the involvement of CD74 in H. pylori adhesion, we examined how CD74 down-regulation when using siRNA affected this adhesion and cytokine production (Fig. 3A). As shown in Fig. 3B, H. pylori adhesion was significantly suppressed by 20% in CD74-silenced cells as compared with the siRNA-transfected control cells. In contrast, IL-8 production was unexpectedly increased in CD74-silenced cells (Fig. 4). It has recently been suggested that MIF up-regulated both CD74 expression and that of the epidermal growth factor receptor (EGFR) which in turn phosphorylated ERK1/2. Furthermore, Kim et al. have recently reported that NCI-N87 cells exhibited the highest expression level of EGFR when compared with several gastric carcinoma cell lines. MEK inhibitor PD98059 (MEK1/2 inhibitor) reduced the IL-8 production in both CD74-silenced and control cells which had been stimulated with H. pylori, while the EGFR tyrosine kinase inhibitor, AG1478, reduced it only in the former cells (Fig. 4). CD74 deficiency did not affect either the spontaneous or H. pylori-induced MIF release level (data not shown).

Discussion

CD74 was detected on gastric epithelial cells and shown to be a new receptor of H. pylori. CD74 is a nonpolymorphic type II integral membrane protein that functions on the signaling pathways for malignant B-cell proliferation and survival. Interestingly, it has also been reported as a high-affinity binding protein for the multifunctional pro-inflammatory cytokine, MIF, which binds to the extracellular domain of CD74, and its interaction with CD44 is required to activate both CD74-silenced and control cells which had been stimulated with H. pylori, while the EGFR tyrosine kinase inhibitor, AG1478, reduced it only in the former cells (Fig. 4). CD74 deficiency did not affect either the spontaneous or H. pylori-induced MIF release level (data not shown).
In this context, we have previously reported that citrus compounds including auraptene exhibited significant inhibitory activity toward CD74 expression at the protein level. Takeda et al. have recently shown that auraptene exhibited anti-*H. pylori* colonization activity in Mongolian gerbils. In this present study, auraptene inhibited *H. pylori* adhesion to NCI-N87 cells by only 27% (Fig. 2A). As already mentioned, the adhesion of *H. pylori* to gastric epithelial cells does not only depend on CD74, but also on many other adhesins and their receptors. However, this coumarin was able to abolish *H. pylori* adhesion-dependent IL-8 production (Fig. 2B), suggesting that this effect, at least in part, could be associated with CD74 suppression. On the other hand, RNAi for CD74 significantly reduced the rate of *H. pylori* adhesion by 22% (Fig. 3B). Our present findings suggest that the mechanism by which auraptene mitigated gastritis was associated, at least in part, with CD74 repression.

IL-8 plays a crucial role in *H. pylori*-associated gastritis by recruiting, activating and infiltrating neutrophils to the site of infection. In addition to its chemotactic potential, it is notable that the level of IL-8 in gastric mucosa is associated with the histological severity in patients with *H. pylori*-induced gastritis. Although CD74 is one of the mediators of *H. pylori* for promoting IL-8 production, other mediators have also been suggested in many studies. For example, several
The MAPK cascade may therefore be responsible for the transcription factors and the resulting expression of IL-8. Reports have proposed that *H. pylori* inoculated CagA into gastric epithelial cells to induce the phosphorylation of MEK, Src and SHP-2, thereby promoting IL-8 production. On the other hand, Tabassam et al. have proposed that although the activation of EGFR/P3K signaling, at least in part, positively regulated *H. pylori*-mediated IL-8 production, CagA was involved in EGFR-dependent IL-8 production. EGFR is over-expressed in various cancer cells and involved in pro-inflammatory responses and procarcinogenic events including cell proliferation, migration, aggregation, and invasion. The expression of this receptor is well known for many cancer tissues including gastric cancer.

In the present study, CD74 down-regulation by siRNA did not suppress, but rather increased IL-8 production when co-cultured with *H. pylori* (Fig. 4). Several studies so far have suggested that *H. pylori* or urease binding to CD74 increased IL-8 production, while *H. pylori* infection induced the expression and activation of EGFR in gastric epithelial cells. Furthermore, MIF appears to induce EGFR phosphorylation through multiple receptors, e.g., CD74, CXCR2, and CXCR4. We have therefore hypothesized that MIF would be associated not only with serum starvation-induced CD74 expression but also that of EGFR. Both PD98059 (the MEK1/2 inhibitor) and AG1478 (the EGFR tyrosine kinase inhibitor) significantly inhibited IL-8 production by *H. pylori* in CD74-silenced cells compared with the control in this study. Previous work has suggested that ERK1/2 played a central role on the signaling pathway linking the activation of EGFR and increased IL-8 expression. Kim et al. have shown that treating AGS cells with transfected with mutant genes for Ras, c-Jun and Ik-Ba with MAPK inhibitors led to the suppression of these *H. pylori*-induced transcription factors and the resulting expression of IL-8. The MAPK cascade may therefore be responsible for inducing chemokine expression through NF-κB and activator protein-1 (AP-1) in *H. pylori*-infected gastric epithelial cells. Interestingly, Kawabata et al. have demonstrated that auraptene strongly dephosphorylated constitutively activated ERK1/2 in HT-29 human colon adenocarcinoma cells. This is consistent with our present findings (Fig. 1B). Dual-specificity phosphatases (DUSP) are a heterogeneous group of protein phosphatases that can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within the one substrate. DUSP can be divided into six subgroups including cell proliferation, migration, aggregation, and invasion. The expression of this receptor is well known for many cancer tissues including gastric cancer.

Fig. 4. Effects of CD74 Knockdown and Specific Inhibitors on *H. pylori*-Increased IL-8 Production in NCI-N87 Gastric Carcinoma Cells. Cells were treated with the control or CD74 siRNA for 7 h, and then the medium were replaced with RPMI1640 including 10% FBS. After 48 h, the cells were exposed to *H. pylori* for 24 h with DMSO, PD98059 (PD: MEK inhibitor, 50 μM) or AG1478 (AG: EGFR tyrosine kinase inhibitor, 10 μM). The concentration of IL-8 in the supernatant was determined by ELISA as described in the Materials and Methods section. The experiments were independently conducted at least three times, and the data are presented as the mean ± SD. Bars not sharing a letter differ, p < 0.05, by Tukey’s multiple-comparison test.

Fig. 5. Proposed Molecular Mechanisms by Which Auraptene Suppresses *H. pylori* Adhesion and IL-8 Production in NCI-N87 Cells.

The adhesion of *H. pylori* to NCI-N87 cells is partially mediated by cell surface CD74. This attachment may activate the MEK/ERK pathway for increased IL-8 production. Auraptene, on the other hand, inactivates ERK1/2 which leads to the down-regulation of both CD74 and IL-8. In contrast, CD74 knockdown by siRNA did not, but rather potentiated IL-8 production. This could be related to the possibility that MIF, a CD74 ligand, may bind to such a surrogate receptor as EGFR. When CD74 is abolished by siRNA, MIF binding to EGFR has previously been shown to activate ERK/IL-8.
been reported to dephosphorylate ERK1/2. In any case, our results suggest that EGFR was partly involved in MIF-induced ERK1/2 activation and IL-8 production (Fig. 5), although other factors such as CagA may also activate ERK1/2 as already mentioned. As illustrated in Fig. 5, while CD74 mediated H. pylori-induced IL-8 production via the MEK/ERK pathway, MIF-activated EGFR may play a similar role in IL-8 production when CD74 is down-regulated.

In conclusion, our results show that citrus auraptene notably suppressed CD74 expression as well as H. pylori adhesion and IL-8 production by regulating ERK1/2. To the best of our knowledge, this is the first report describing a food phytochemical that has the potential for regulating H. pylori. This study was supported in part by grant-aid from the Japan Society for the Promotion of Science for young scientists (H.S.).

Acknowledgments

This study was supported in part by grant-aid from the Council for Advancement of Fruit Tree Science, by grant-aid for cancer research from the Ministry of Health, Labor and Welfare of Japan (A.M.), and by grant-aid for research fellowships from the Japan Society for the Promotion of Science for young scientists (H.S.).

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