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β-carotene Inhibits Expression of c-Myc and Cyclin E in *Helicobacter pylori*-infected Gastric Epithelial Cells

SHORT COMMUNICATION

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Background: *Helicobacter pylori* infection is a major risk factor in the development of gastric cancer. *H. pylori* infection of gastric epithelial cells increases the levels of reactive oxygen species (ROS), activates oncogenes, and leads to β-catenin-mediated hyper-proliferation. β-Carotene reduces ROS levels, inhibits oxidant-mediated activation of inflammatory signaling and exhibits anticancer properties. The present study was carried out to determine if β-carotene inhibits *H. pylori*-induced cell proliferation and the expression of oncogenes c-myc and cyclin E by reducing the levels of β-catenin and phosphorylated glycogen synthase kinase 3β (p-GSK3β).
Methods: Gastric epithelial AGS cells were pre-treated with β-carotene (5 and 10 μM) for 2 hours prior to *H. pylori* infection and cultured for 6 hours (for determination of the levels of p-GSK3β, GSK3β, and β-catenin) and 24 hours (for determination of cell viability and protein levels of c-myc and cyclin E). Cell viability was determined by the MTT assay and protein levels were determined via western blot-based analysis.
Results: β-Carotene inhibited *H. pylori*-induced increases in the percentage of viable cells, phosphorylated GSK3β (p-GSK3β), and the levels of β-catenin, c-myc and cyclin E.
Conclusions: β-Carotene inhibits *H. pylori*-induced hyper-proliferation of gastric epithelial cells by suppressing β-catenin signaling and oncogene expression.
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Key Words: Beta carotene, Beta catenin, *Helicobacter pylori*, Epithelial cells, Oncogenes

INTRODUCTION

Gastric cancer, which is one of the most common cancers worldwide, is induced by colonization of the stomach by the bacterial pathogen *Helicobacter pylori*. Studies have shown that *H. pylori* infection of gastric epithelial cells increases the levels of reactive oxygen species (ROS) which are produced by NADPH oxidase, activates oncogenes, and leads to hyper-proliferation and tumor formation [1-5]. ROS, in turn, activate Janus-activator kinases (Jak)/Stat [2] to increase the expression of the β-catenin-regulated gene c-myc, and the c-myc-regulated gene cyclin E [3,4]. The transcription factor c-myc inhibits cell growth arrest and promotes cell proliferation [6] whereas cyclin E regulates the G1 phase of the cell cycle [7]. The expression of c-myc is induced by β-catenin [8]. Several stuides demonstrated that β-catenin signaling mediates the expression of cyclin E in several diffrent cell lines [9]. Thererfore, in the present study, cyclin E was determined as a target gene of β-catenin.

β-Catenin signaling is activated by ROS, which are present at higher levels in cancer cells than in normal cells [10-13]. Cellular β-catenin levels are reduced via the ubiquitin-proteosome system consisting of glycogen synthase kinase 3β (GSK3β) [14] and remains low in normal cells. However, phosphorylation of GSK3β to form p-GSK3β inactivates GSK3β for destruction of β-catenin. Therefore, phosphorylation of GSK3β inhibits the degradation of β-catenin, which leads to the accumulation β-catenin in the cells. At high levels, β-catenin translocates into nucleus, leading to induction of β-catenin-regulated oncogenes, such as c-myc and cyclin E.

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β-Carotene is an antioxidant carotenoid found in orange colored fruits and vegetables such as carrots and sweet potatoes. It suppressed cancer cell growth [15,16]. We previously demonstrated that β-carotene reduced ROS and suppressed oxidant-sensitive transcription factors (NF-κB, AP-1) and expression of inducible nitric oxide and cyclooxygenase-2 in *H. pylori*-infected gastric epithelial cells [17].

The present work was carried out to determine if antioxidant β-carotene inhibits *H. pylori*-induced cell proliferation and the expression of oncogenes c-myc and cyclin E by reducing the levels β-catenin and active glycogen synthase kinase 3β (p-GSK3β).

In the text below, we demonstrate that β-carotene inhibits *H. pylori*-induced hyper-proliferation by suppressing expression of β-catenin and phosphorylation of GSK3β, and reducing the protein levels of c-myc and cyclin E in gastric epithelial cells.

MATERIALS AND METHODS

1. Cell line and culture conditions

The human gastric epithelial AGS cells (gastric adenocarcinoma, ATCC CRL 1739; American Type Culture Collection, Rockville, MD, USA) were grown as described previously [18]. *H. pylori* cells (NCTC 11637, American Type Culture Collection) were grown on chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C, under microaerophilic conditions, using an anaerobic chamber (BBL Campy Pouch® System; Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA). The *H. pylori* strain NCTC 11637 expresses the proteins of CagA, VacA, cytotoxin and urease [19].

AGS cells were seeded overnight to reach 80% confluency. The *H. pylori* was harvested and suspended in antibiotic-free RPMI 1640 medium supplemented with 10% FBS, and then added to the AGS cell culture at a 50 : 1 ratio of bacterium : AGS cells. AGS cells (1.0×10^5/mL) were pre-treated with 5 or 10 μM β-carotene (dissolved in tetrahydrofuran [17]) for 2 hours prior to the addition of the *H. pylori*. The infected cells were incubated for an additional 6 hours for determination of the levels of p-GSK3β, GSK3β, and β-catenin or 24 hours for determination of cell viability and the levels of c-myc and cyclin E) in the presence of *H. pylori*. The 5 and 10 μM β-carotene concentrations used in the pre-treatment are reportedly nontoxic [20,21].

2. Cell viability and Western blot analysis

Cell viability was determined using MTT (thiazoyl blue; Sigma-Aldrich, St. Louis, MO, USA) as described previously [22]. The results were expressed as the mean ± SE of 3 different experiments. For each experiment, the number of each group was 4 (n = 4 per each group). A *P*-value of 0.05 or less was considered statistically significant.

For Western blot analysis, the whole-cell extracts were prepared as previously described [22] as were the Western blots [23]. The proteins were detected using antibodies for p-GSK3β (#5558; Cell Signaling Technology, Danvers, MA, USA), GSK3β (sc-7291; Santa Cruz Biotechnology, Dallas, TX, USA), β-catenin (610154; BD Transduction Laboratories, Franklin Lakes, NJ, USA), c-myc (sc-40, Santa Cruz Biotechnology), cyclin E (sc-481, Santa Cruz Biotechnology), and the loading control actin (sc-1615, Santa Cruz Biotechnology) in TBS-T solution containing 3% dry milk, and incubated overnight at 4°C. After washing with TBS-T, the primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-mouse, anti-rabbit, anti-goat), and visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology). The ratio of the band densities corresponding to β-catenin, c-myc and cyclin E to that of the loading control actin were calculated. The ratio of the p-GSK3β band density to the band density of total GSK3β was calculated. Values are expressed as the mean ± SE of 4 per each group. A *P*-value of 0.05 or less was considered statistically significant.

3. Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Newman–Keul's tests. The results were expressed as the mean ± SE of 3 different experiments. For each experiment, the number of each group was 4 (n = 4 per each group). A *P*-value of 0.05 or less was considered statistically significant. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

The impact of β-carotene on AGS cell viability following infection with *H. pylori* was measured as a function of β-carotene concentration. The results are reported in Figure 1A. The viability of the AGS cells uninfected with *H. pylori* (see figure column labeled "None") was found to be higher than that of infected cells (figure column labeled "Control"). The findings of the present work show that the treatment of the AGS cells with β-carotene prior to *H. pylori* infection reduces cell viability in a dose-dependent manner.

Next, the effect of β-carotene on the cellular levels of β-catenin and GSK3β in its phosphorylated (p-GSK3β) and total (GSK3β) forms was examined. The results are reported in Figure 1B. The

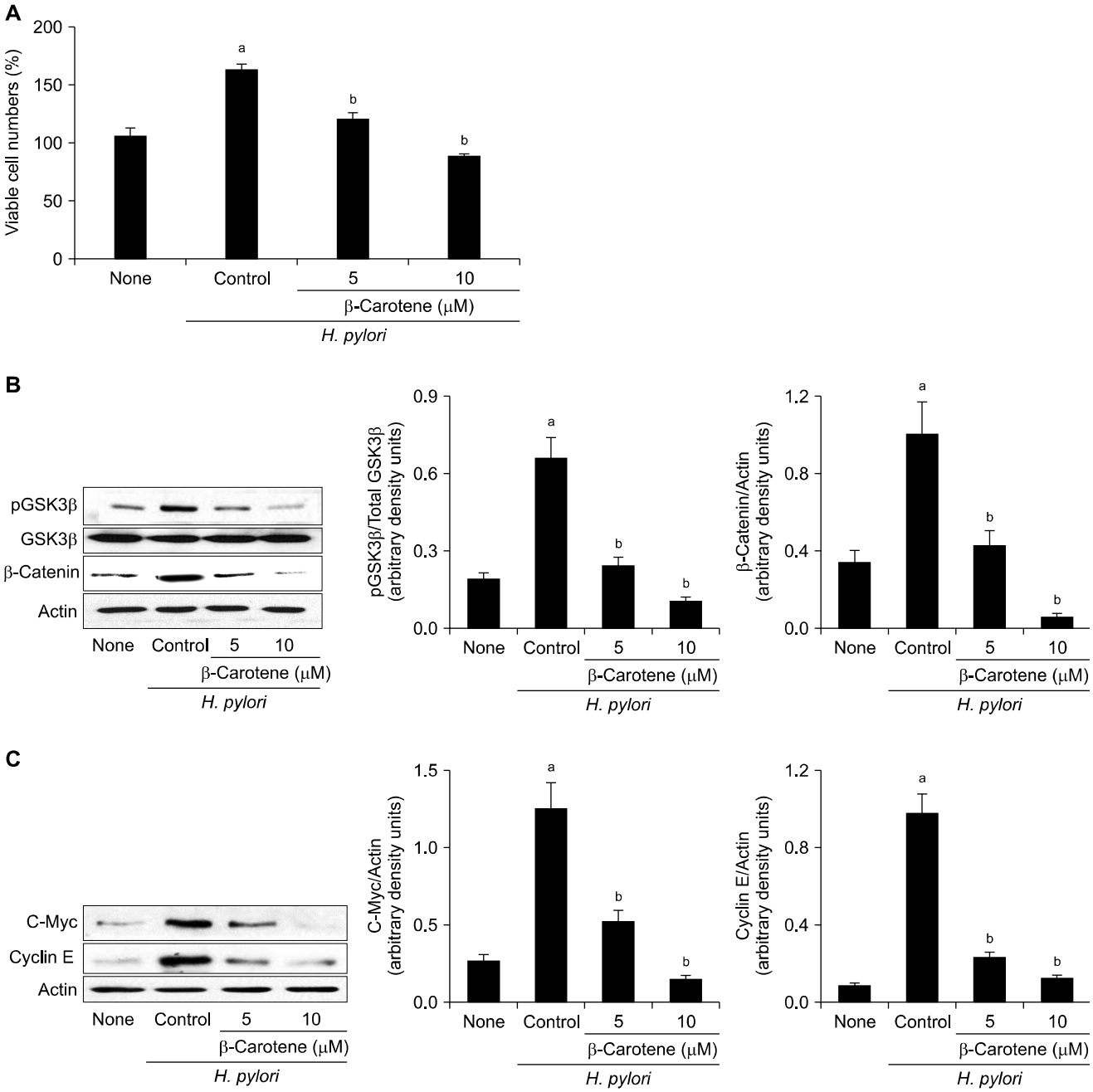

Figure 1. Effect of β-carotene on cell viability, phosphorylation of GSK3β, and expression of β-catenin, c-myc, and cyclin E in gastric epithelial cells with *Helicobacter pylori* infection. Uninfected cells (column "None"), cells with *H. pylori* infection in the absence of β-carotene (column "Control") and in the presence of 5 μM or 10 μM β-carotene (columns β-carotene 5 and 10, respectively). (A) The percentage of viable cells. $^{a}P < 0.05$ vs. "None"; $^{b}P < 0.05$ vs. "Control". All values were expressed as mean ± SE of 3 different experiments (n = 4 per group for each experiment). (B) Left panel: Western blot of β-catenin, glycogen synthase kinase 3β (GSK3β) and phosphorylated glycogen synthase kinase 3β (p-GSK3β). Center panel: ratio of p-GSK3β/total GSK3β calculated from the Western blot. Right panel: the amount of β-catenin calculated from the Western blot and reported in ratio to the gel loading control actin. (C) Left panel: Western blot of c-myc and cyclin E. Center panel: the amount of c-myc calculated from the Western blot and reported in ratio to the gel loading control actin. Right panel: the amount of cyclin E calculated from the Western blot and reported in ratio to the gel loading control actin. $^{a}P < 0.05$ vs. "None"; $^{b}P < 0.05$ vs. "Control". Values are expressed as the mean ± SE of 4 per each group. A *P*-value of 0.05 or less was considered statistically significant.

results show that the ratio of p-GSK3β to total GSK3β is increased ~3.5-fold as a result of *H. pylori* infection (Fig. 1B, center panel; "Control" vs. "None"). Importantly, pretreatment of the AGS cells with β-carotene at low concentrations (5 and 10 μM) prior to infection reduced the ratio of p-GSK3β/GSK3β in a dose-dependent manner. The cellular level of β-catenin was increased ~2.5-fold as a result of *H. pylori* infection (Fig. 1B, right panel; "Control" vs. "None"). β-Carotene treatment reduced *H. pylori*-induced increase of β-catenin in AGS cells concentration-dependently.

Lastly, the effect of β-carotene on the cellular levels of c-myc and cyclin E was measured and the results obtained are shown in Figure 1C. *H. pylori* infection raised the cellular level of c-myc ~4-fold and that of cyclin E ~8.5-fold. (Fig. 1C; "Control" vs. "None" for center panel and right panel, respectively). β-Carotene diminished the *H. pylori*-induced increases in c-myc and cyclin E in dose-dependent manner.

DISCUSSION

Our results show that β-carotene at low concentration (5 and 10 μM) protects gastric epithelial cells from increased proliferation caused by cancer-promoting *H. pylori* infection (Fig. 1A). It is, however, important to note here that we have previously reported that the treatment of gastric epithelial cells with β-carotene at a 10-fold higher concentration range (50 and 100 μM) than that used in the present study, induces an increase in ROS, a decrease in the DNA repair protein complex Ku, activation of caspase-3, and an increase in apoptosis [24]. In addition, it has been observed that at high concentration β-carotene increases the level of ROS in adenocarcinoma and leukemia cells [25,26]. Together, these findings indicate that the physiological effects of β-carotene can vary with concentration range and therefore, that dosage will be an important consideration in the use of β-carotene in cancer treatment.

The effects of β-carotene at low concentration used in the present study changed the physiology of the gastric epithelial cells resulting from *H. pylori* infection. Specifically, the increase in the level of β-catenin observed upon *H. pylori* infection (Fig. 1B) induces the target gene c-myc thereby increasing the level of c-myc, consistent with our observations (Fig. 1C). Furthermore, because c-myc induces the synthesis of cyclin E, *H. pylori* infection results in higher levels of cyclin E, as is confirmed by the results shown in Figure 1C. Importantly, treatment of the cells with β-carotene prior to infection with *H. pylori* prevented these increases in c-myc and cyclin E.

To gain insight into how the level of β-catenin is increased by *H. pylori* infection, we measured the cellular level of the β-catenin regulator GSK3β. The observation that p-GSK3β was increased by *H. pylori* infection (Fig. 1B) is consistent with increased levels of β-catenin. Because GSK3β phosphorylation prevents the destruction of its substrate β-catenin, blocking β-catenin degradation results in accumulation of cellular β-catenin. Treatment of the cells with β-carotene prior *H. pylori* infection reduces p-GSK3β and hence, the elevation in the levels of β-catenin, c-myc and cyclin E that accompanies the infection. We have previously demonstrated that β-carotene reduces ROS and suppresses the activities of the transcription factor NF-κB in *H. pylori*-infected gastric epithelial cells [17]. In addition, other studies have shown that ROS activates β-catenin signaling via NF-κB activation [27,28] and that β-carotene inhibits the activation of NF-κB [29]. Inhibition of the ROS-generating enzyme NADPH oxidase suppresses *H. pylori*-induced NF-κB activation and β-catenin signaling [30]. Therefore, it is reasonable to attribute the β-carotene-induced inhibition of GSK3β phosphorylation and β-catenin accumulation, observed in the present study, to the antioxidant activity of β-carotene.

In conclusion, our findings support the proposal that consumption of β-carotene-rich food may prevent the development of *H. pylori*-associated gastric disorders by suppressing oncogene expression and gastric cell hyper-proliferation. Future work will examine the impact of β-carotene on the accumulation of β-catenin within the nucleus of *H. pylori*-infected gastric epithelial cells as well as the effect of β-carotene on the viability of the infectious agent *H. pylori*. In addition, it is essential to determine transcriptional activity of β-catenin to prove whether *H. pylori* induces c-myc and cyclin E through the β-catenin activation for future study.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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