Transgenic cyclooxygenase-2 expression and high salt enhanced susceptibility to chemical-induced gastric cancer development in mice

Wai K.Leung1,∗, Kai-chun Wu1, Christine Y.P.Wong1, Alfred S.L.Cheng1, Arthur K.K.Ching2, Anthony W.H.Chan1, Wilson W.S.Chong1, Minnie Y.Y.Go1, Jun Yu1, Ka-Fai To1, Xin Wang3, Y.L.Chui2, D.M.Fan3 and Joseph J.Y.Sung1

1Institute of Digestive Disease and Li Ka Shing Institute of Health Sciences, 2Clinical Immunology Unit, The Chinese University of Hong Kong, Hong Kong, China and 3Institute of Digestive Diseases, Fourth Military Medical University, Xi’an 710032, China

∗To whom correspondence should be addressed. Department of Medicine & Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. Tel: +852 2632 3173; Fax: +852 2637 3852, Email: dr_wkleung@alumni cuhk.net

Carcinogenesis vol.29 no.8 pp.1648–1654, 2008
doi:10.1093/carcin/bgn156
Advance Access publication July 7, 2008

Cyclooxygenase (COX)-2 overexpression is involved in gastric carcinogenesis. While high-salt intake is a known risk factor for gastric cancer development, we determined the effects of high salt on gastric chemical carcinogenesis in COX-2 transgenic (TG) mice. COX-2 TG mice were developed in C57/BL6 strain using the full-length human cox-2 complementary DNA construct. Six-week-old COX-2 TG and wild-type (WT) littermates were randomly allocated to receive alternate week of N-methyl-N-nitrosourea (MNU, 240 p.p.m.) in drinking water or control for 10 weeks. Two groups of mice were further treated with 10% NaCl during the initial 10 weeks. All mice were killed at the end of week 50. Both forced COX-2 overexpression and high-salt intake significantly increased the frequency of gastric cancer development in mice as compared with WT littermates treated with MNU alone. However, no additive effect was observed on the combination of high salt and COX-2 expression. We further showed that MNU and high-salt treatment increased chronic inflammatory infiltrates and induced prostaglandin E2 (PGE2) production in the non-cancerous stomach. Whereas high-salt treatment markedly increased the expression of inflammatory cytokines (tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β and IL-6) in the gastric mucosa, COX-2 overexpression significantly altered the cell kinetics in the MNU-induced gastric cancer model. In conclusion, both high salt and COX-2 overexpression promote chemical-induced gastric carcinogenesis, possibly related to chronic inflammation, induction of PGE2, disruption of cell kinetics and induction of inflammatory cytokines.

Introduction

Gastric cancer is the second commonest cancer in the world. It is particularly prevalent in East Asian countries where it remains the leading cancer killer (1). While advanced gastric cancer is associated with high morbidity and mortality, early detection and prevention is the best approach in reducing gastric cancer incidence and its related mortality. Despite the strong link between Helicobacter pylori infection and gastric cancer development, recent intervention trials fail to show any significant effects of H.pylori eradication alone in preventing gastric cancer development in human subjects (2–5). On the other hand, there are compelling epidemiological data to suggest that long-term use of non-steroidal anti-inflammatory drugs is associated with a significant reduction in gastric cancer risk (6). This protection is largely attributed to the inhibition of cyclooxygenase (COX) enzyme, particularly the COX-2 isofrom (7). In this regard, we have shown previously that treatment with celecoxib, a specific COX-2 inhibitor, prevents gastric cancer development in a rodent model of gastric carcinogenesis (8). Nonetheless, we found that the chemopreventive effect of celecoxib appears to be unrelated to the degree of COX and prostaglandin inhibition in that study (9). Hence, it is suggested that non-COX-mediated pathway may be involved in celecoxib-mediated chemoprevention. In addition to established cancer, COX-2 expression was demonstrated in other stages of H.pylori-associated gastric carcinogenesis including chronic gastritis, intestinal metaplasia and dysplasia (10,11). Although enhanced COX-2–prostaglandin E synthase pathway was shown to induce hyperplastic tumor development (12), the role of COX-2 *per se* on gastric cancer development remains elusive. In particular, it remains unknown whether COX-2 initiates or promotes gastric tumor development.

Apart from *H.pylori* infection, high dietary salt intake is frequently linked to gastric cancer development. Epidemiological studies showed that high dietary salt intake is a significant risk factor for human gastric cancer development even after adjustment for *H.pylori* infection and atrophic gastritis (13). In mouse model, high salt has been shown to induce gastric epithelial hyperplasia, parietal cell loss as well as *H.pylori* colonization (14). Experimental studies also showed that salt promotes gastric carcinogenesis in animals after chemical carcinogen treatment (8,15). Recently, Kato et al. (16) further showed that high salt dose dependently promotes gastric chemical carcinogenesis in *H.pylori*-infected Mongolian gerbils.

In this study, we sought to determine the effects of forced COX-2 expression alone and in combination with high-salt intake on gastric chemical carcinogenesis in a COX-2 transgenic (TG) mouse model.

Materials and methods

Animals

COX-2 TG mice were generated by cloning of the full-length human cox-2 complementary DNA downstream of the cytomegalovirus promoter of the pcDNA3.1 plasmid. The sequence of the clone was confirmed by DNA sequencing. The linearized and purified CMV-cox-2 DNA transgene was used for microinjection into pronuclei of fertilized C57BL/6 F1 oocytes. Survived embryos were transferred into the oviducts of pseudopregnant female mice. In this study, we sought to determine the effects of forced COX-2 expression alone and in combination with high-salt intake on gastric chemical carcinogenesis in a COX-2 transgenic (TG) mouse model.

Abbreviations: COX, cyclooxygenase; IFN, interferon; IL, interleukin; MNU, N-methyl-N-nitrosourea; mPGES, microsomal prostaglandin E synthase; PCR, polymerase chain reaction; PGE2, prostaglandin E2; TG, transgenic; TNF, tumor necrosis factor; WT, wild-type.

© The Author 2008. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
Histopathology and immunohistochemistry

Gastric specimens were formalin fixed and paraffin embedded for histological examination. Sections of 5 μm were stained with hematoxylin and eosin for histological diagnosis by an experienced pathologist who was unaware of the treatment allocation and genetic background of the mice. Gastric cancer was defined as the unequivocal presence of invasive adenocarcinoma in the glandular stomach (Figure 3). Severity of gastric inflammation including acute inflammation, chronic inflammation, atrophy and intestinal metaplasia was determined based on the updated Sydney classification (19).

COX-2 immunostaining was performed by the ABComplex/HRP method (Dako, Carpinteria, CA) using the anti-COX-2 rabbit monoclonal antibody (NeoMarkers, Fremont, CA). Heat-induced antigen recovery was performed before the immunoreactions in a moist chamber. The reactions were visualized with diaminobenzidine substrate and counterstained with hematoxylin solution.

Quantification of apoptotic and proliferation index

Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling and proliferation was quantitated by Ki-67 immunostaining as described previously (9). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling assay was performed with DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) as suggested by the manufacturer. Ki-67 immunostaining was performed by the LAB-SA Detection System (Zymed, S. San Francisco, CA) using the anti-Ki-67 monoclonal antibody (Lab Vision, Fremont, CA). Heat-induced antigen recovery was performed and reactions were visualized with diaminobenzidine substrate counterstained with hematoxylin solution. All histological sections were examined in high-power fields (400×). A random starting field was selected and then other field was examined for a total of >1000 gastric epithelial cells.

Fig. 1. COX-2 expression in stomach of COX-2 TG mice. (A and B) Intense COX-2 immunoreactivity in the stomach of TG mice as compared with WT mice (magnification ×200). (C) Western Sydney further confirmed the overexpression of COX-2 in stomach of TG (lanes 1 and 2) as compared with WT (lanes 3-6). There was high COX-2 expression in both tumors as well as normal stomach of the TG mice. (D) PGE2 level was also significantly higher in the stomach of TG mice as compared with WT mice (P = 0.016). Values are shown as mean ± SD (error bar).

Fig. 2. Study design. Six-week-old WT and Cox-2 TG mice were allocated to six different treatment groups. Groups A (WT) and D (TG) were control groups. Groups B (WT) and E (TG) received alternate week of MNU at 240 p.p.m. for 10 weeks. In addition to MNU, Groups C (WT) and F (TG) received 10% NaCl weekly during the initial 10 weeks. All mice were killed at the end of week 50.
Real-time reverse transcription–PCR

Total RNA was extracted from gastric tissues by using RNA Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Five micrograms of total RNA was reverse transcribed into complementary DNA. RNA levels of inflammatory cytokines, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6 and IL-10, were quantified by real-time reverse transcription–PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The sequences of primers were listed in supplementary Table 1 (available at Carcinogenesis Online). β-Actin served as an internal control for total complementary DNA samples. Samples were amplified using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All PCRs were run in triplicates to ensure reproducibility.

Statistical analysis

Numerical data were presented as mean ± SD unless otherwise specified. Multiple group comparison was made by one-way analysis of variance with Bonferroni’s adjustment. All statistical analysis was performed by GraphPad InStat (ver 3.0, GraphPad Software, San Diego, CA). A two-sided P value of <0.05 was considered statistically significant.

Results

Verification of COX-2 expression in TG mice

The TG mice line with COX-2 overexpression in the gastric epithelium was selected for this study. There was a marked increase in COX-2 expression in the glandular stomach of TG mice as shown by immunohistochemistry (Figure 1A and B) and western blot (Figure 1C). In keeping with COX-2 expression, the level of PGE2 in the gastric mucosa of the TG mice was ~4-fold higher than that of WT mice (P = 0.016; Figure 1D). There was no significant histological change in the gastric mucosa of TG mice.

Gastric cancer development in COX-2 TG mice

At the end of week 50, none of the control WT (Group A) or control COX-2 TG (Group D) littermates developed gastric cancer in the absence of MNU treatment. In contrast, gastric cancer was detected in 25% of WT mice treated with MNU alone (Group B). Additional treatment with 10% NaCl (Group C) further increased the rate of gastric cancer to 46.9% in WT mice (P = 0.014 versus Group B; Figure 3). After MNU treatment, COX-2 TG mice (Group E) had a markedly higher gastric cancer rate than WT mice (47.5%; P = 0.007 versus Group B). Addition of 10% NaCl, however, did not further increase the gastric cancer incidence in COX-2 TG mice (48.1%, Group F).

Gastric inflammation scores

The pattern of chronic inflammatory infiltrates appears to parallel the rates of gastric cancer in different groups of mice (Figure 4). While there were no chronic inflammatory infiltrates in WT and TG mice alone, the addition of MNU or 10% NaCl significantly induced chronic inflammatory cells infiltration (P = 0.009). Similar trend was also observed in the atrophy and metaplasia scores but the difference did not reach statistical significance. The acute inflammatory scores did not appear to correlate with gastric cancer incidences in different groups of mice.

PGE2 levels and COX-2 expression

We next determined the PGE2 levels in the gastric mucosa of different groups of mice. The PGE2 levels in the normal non-cancerous gastric mucosa of different groups are shown in Figure 5. There was a significant difference in PGE2 production among different groups (P = 0.003). While COX-2 TG mice have a marked induction in PGE2 production when compared with WT mice, treatment with MNU increased the PGE2 levels in WT by >5-fold. Similar induction of PGE2 was not detected in COX-2 TG mice treated with MNU (Group E). Notably, high salt further increased the PGE2 production by ~2-fold in both WT and COX-2 TG mice treated with MNU (Groups C and F). There was, however, no significant difference in

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>WT</th>
<th>WT</th>
<th>TG</th>
<th>TG</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10% NaCl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Statistical analysis of gastric inflammation scores

Results

Verification of COX-2 expression in TG mice

The TG mice line with COX-2 overexpression in the gastric epithelium was selected for this study. There was a marked increase in COX-2 expression in the glandular stomach of TG mice as shown by immunohistochemistry (Figure 1A and B) and western blot (Figure 1C). In keeping with COX-2 expression, the level of PGE2 in the gastric mucosa of the TG mice was ~4-fold higher than that of WT mice (P = 0.016; Figure 1D). There was no significant histological change in the gastric mucosa of TG mice.

Gastric cancer development in COX-2 TG mice

At the end of week 50, none of the control WT (Group A) or control COX-2 TG (Group D) littermates developed gastric cancer in the absence of MNU treatment. In contrast, gastric cancer was detected in 25% of WT mice treated with MNU alone (Group B). Additional treatment with 10% NaCl (Group C) further increased the rate of gastric cancer to 46.9% in WT mice (P = 0.014 versus Group B; Figure 3). After MNU treatment, COX-2 TG mice (Group E) had a markedly higher gastric cancer rate than WT mice (47.5%; P = 0.007 versus Group B). Addition of 10% NaCl, however, did not further increase the gastric cancer incidence in COX-2 TG mice (48.1%, Group F).

Gastric inflammation scores

The pattern of chronic inflammatory infiltrates appears to parallel the rates of gastric cancer in different groups of mice (Figure 4). While there were no chronic inflammatory infiltrates in WT and TG mice alone, the addition of MNU or 10% NaCl significantly induced chronic inflammatory cells infiltration (P = 0.009). Similar trend was also observed in the atrophy and metaplasia scores but the difference did not reach statistical significance. The acute inflammatory scores did not appear to correlate with gastric cancer incidences in different groups of mice.

PGE2 levels and COX-2 expression

We next determined the PGE2 levels in the gastric mucosa of different groups of mice. The PGE2 levels in the normal non-cancerous gastric mucosa of different groups are shown in Figure 5. There was a significant difference in PGE2 production among different groups (P = 0.003). While COX-2 TG mice have a marked induction in PGE2 production when compared with WT mice, treatment with MNU increased the PGE2 levels in WT by >5-fold. Similar induction of PGE2 was not detected in COX-2 TG mice treated with MNU (Group E). Notably, high salt further increased the PGE2 production by ~2-fold in both WT and COX-2 TG mice treated with MNU (Groups C and F). There was, however, no significant difference in
the expression of COX-2 protein after MNU and/or NaCl treatment in both WT and TG mice (data not shown).

Inflammatory cytokine expressions
Apart from PGE2, we also determined the inflammatory cytokine expressions in the normal non-cancerous gastric epithelium of different groups of mice (Figure 6). The levels of five inflammatory cytokines including TNF-α, IFN-γ, IL-1β, IL-6 and IL-10 were determined by quantitative reverse transcription-PCR. There was a marked difference in the expression levels of the five inflammatory cytokines among the six different groups ($P < 0.0001$). Specifically, treatment with 10% NaCl significantly induced the expression of TNF-α, IFN-γ, IL-1β and IL-6 in WT mice treated with MNU (Group C; $P < 0.001$ versus Groups A, B, D and E). In COX-2 TG treated with MNU (Group F), addition of 10% NaCl enhanced all the five cytokines expression ($P < 0.01$ versus Groups A, B, D and E).

Apoptosis and proliferation changes
We also compared the apoptosis and proliferation index in the non-cancerous stomach of the mice (Figure 7A). There was a significant difference in the apoptotic ($P = 0.003$; Figure 7B) and proliferation ($P = 0.03$; Figure 7C) indexes among different treatment groups. COX-2 TG (Group D) or treatment with salt (Groups C and F) did not appear to significantly alter the basal apoptotic and proliferation index of gastric epithelium. However, the addition of MNU to COX-2 TG mice resulted in the highest apoptotic ($P < 0.01$ versus Group A control) and proliferation index ($P < 0.05$ versus Group A control) among all six groups.

Discussion
The current study showed that forced COX-2 expression in mice alone fails to induce gastric cancer development but rather enhances gastric cancer formation induced by chemical carcinogen MNU. This is the first piece of evidence to demonstrate the temporal effect of COX-2 on gastric carcinogenesis that appears to be more on promotion rather than initiation of cancer development. This is in keeping with our previous observations that COX-2 overexpression is detected in different stages of H. pylori-associated gastric carcinogenesis including chronic gastritis, glandular atrophy, intestinal metaplasia and cancer (10,11). Intuitively, forced overexpression of COX-2 promotes gastric cancer development in the multistep gastric carcinogenesis cascade.

On the other hand, high-salt intake has been suspected to be an important risk factor for gastric cancer. Based on epidemiological evidence, a Joint WHO/FAO Expert Consultation concluded that high-salt intake probably increase the risk of stomach cancer (20). In the current study, we showed that treatment with 10% NaCl for 10 weeks markedly promoted gastric cancer development in WT mice treated with MNU. The percentage of WT mice that developed gastric cancer increased from 25 to 46.9% after high-salt treatment. The effect of salt on cancer promotion in this mouse model is comparable with the effect of COX-2 overexpression, but there was no additive
In the present study, the elevated PGE2 levels can-...tumorous growth. In the present study, the elevated PGE2 levels can-

tor-4 in the gastric epithelial cells, resulting in gastric metaplasia and

and stimulation that was mediated through epithelial Toll-like recep-

further shown that increased PGE2 enhances macrophage infiltration

mice expressing both COX-2 and microsomal prostaglandin E syn-

one of the key prostanoids responsible for gastric carcinogenesis. TG

D and E).

W.K. Leung et al.

WT and TG mice treated with MNU. PGE2 is generally believed to be

induction of inflammatory cytokines in mice treated with salt. We

not be solely explained by COX-2 protein expression alone. We spec-

cifically increased PGE2 production in non-cancerous mucosa of both

animal model. We also found that the addition of high salt signifi-

chronic inflammation contributes to gastric carcinogenesis in this

We next elucidated the procarcinogenic effects of COX-2 and salt

in this MNU model of gastric carcinogenesis. The rates of gastric
cancer in different groups of mice appear to parallel the degree of

chronic inflammation in the stomach, suggesting that chronic inflam-
mation contributes to gastric carcinogenesis in this animal model. We also found that the addition of high salt signifi-

antly increased PGE2 production in non-cancerous mucosa of both

WT and TG mice treated with MNU. PGE2 is generally believed to be

one of the key prostanoids responsible for gastric carcinogenesis. TG

mice expressing both COX-2 and microsomal prostaglandin E syn-
thase (mPGES)-1 developed hyperplastic gastric tumors (12). It was

investigation.

In conclusion, both forced COX-2 overexpression and high-salt

treatment promoted chemical-induced gastric cancer development in

mice. These cancer-promoting effects are possibly contributed by

various factors including chronic inflammation, increased PGE2 pro-
duction, induction of inflammatory cytokines and disruption of cell

kinetics.
Fig. 7. Apoptosis and proliferation index in non-cancerous stomach of mice. (A) Representative sections showing apoptotic nuclei by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (left, arrow) and proliferation by Ki67 immunostaining (right). (B) Apoptotic index was determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling assay. There was a significant difference in apoptotic index among all six treatment groups ($P = 0.003$). The highest apoptotic index was seen COX-2 TG mice treated with MNU ($**P < 0.01$ versus Group A). (C) Proliferation index was determined by Ki67 immunostaining. A significant difference in proliferation index among the six treatment groups was detected ($P = 0.03$). The highest proliferation index was observed in COX-2 TG mice treated with MNU ($P < 0.05$).

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/.

Funding

Joint Research Project Scheme of the Natural Science Foundation of China; Research Grant Council of Hong Kong (N_CUHK420/03).
Acknowledgements

Conflict of Interest Statement: None declared.

References


Received January 22, 2008; revised June 17, 2008; accepted June 22, 2008