Effects of synthetic and natural in vivo inhibitors of β-glucuronidase on azoxymethane-induced colon carcinogenesis in rats

NANAE MORITA1, ZBIGNIEW WALASZEK4, TATSUYA KINJO1,2,4, TADASHI NISHIMAKI2, MARGARET HANAUSEK4, THOMAS J. SLAGA4, HIDEKI MORI3 and NAOKI YOSHIMI1

1Tumor Pathology, and 2Digestive and General Surgery, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215; 3Tumor Pathology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan; 4Department of Pharmacology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA

Received April 14, 2008; Accepted June 2, 2008
DOI: 10.3892/mmr_00000022

Abstract. d-Glucaric acid is a non-toxic natural compound found in many fruits and vegetables. Our previous studies have shown that the β-glucuronidase inhibitor D-glucaric acid is an active metabolite of D-glucaric acid, inhibits chemically-induced tumorigenesis in rodents. D-Glucaric acid has a synthetic precursor, 2,5-di-O-acetyl-D-glucaro-1,4:6,3-dilactone or aceglatone (ACE), known as a postoperative prophylactic agent, and a natural precursor, D-glucurono-γ-lactone (GL). In the present study, we first examined the effect of ACE on the initiation phase of rat colon carcinogenesis induced by 15 mg/kg azoxymethane (AOM) administered 3 times by subcutaneous (s.c.) injection at weeks 1, 2, and 3 of a 5-week short-term experiment. ACE (0.5 or 2%) was administered as a dietary supplement for 5 weeks. At 5 weeks after the initiation of treatment, the formation of aberrant crypt foci (ACF) in the rat groups treated with AOM plus a 0.5 or 2% ACE diet was significantly reduced by 48.6 and 55.3%, respectively, compared to the group administered AOM alone. In a previous study, 0.5 and 2% ACE diets dispensed during AOM treatment had a tendency to decrease AOM-induced colonic tumor incidence. In the present long-term 36-week colon tumorigenesis experiment, GL (0.5 or 2%) administered via the diet during the initiation phase (starting 1 week before the first dose of AOM and ending 1 week after the 3rd dose) did not have any significant effects on tumor incidence. On the other hand, continued post-initiation treatment with ACE (0.5 and 2%) markedly reduced colonic tumor incidence by 70 and 80%, respectively. GL was effective to a similar extent (70% inhibition), but only at a concentration of 2%. We conclude that ACE inhibits the initiation and post-initiation stages of AOM-induced colon carcinogenesis, while GL affects only the post-initiation stages.

Introduction

Colorectal cancer is one of the most common forms of cancer, occurring worldwide. In Japan, colon cancer incidence has shown a marked increase (1). Therefore, research aimed at the prevention of colon cancer, in particular by the identification of cancer chemopreventive agents, is urgently needed (2,3). Our laboratory has examined natural and synthetic compounds, and has provided evidence that newly-extracted and/or previously known products have the potential to prevent the development of chemically-induced tumors in animal models (4-8). It is known that the body detoxifies xenobiotics, i.e., lipid-soluble toxins including certain carcinogenic compounds, by conjugation with glutathione, by sulfation and by glucuronidation, a principal conjugation pathway in all tissues (9). Carcinogenic compounds are conjugated with glucuronic acid in the liver or kidney, then excreted as glucuronides in bile or urine (9). As a result of this process, many carcinogens and tumor promoters are eliminated from the body. However, following the excretion of bile into the gastrointestinal tract, β-glucuronidase, a bacterial enzyme which resides in the gut, may hydrolyze the glucuronic acid conjugate and liberate toxins such as active carcinogens and tumor promoters, reabsorbing them back into the body instead of allowing them to be excreted. The final rate of elimination of toxic chemicals is therefore determined not only by glucuronidation, but also by de-glucuronidation carried out by β-glucuronidase (9,10).

Elevated β-glucuronidase activity is associated with an increased risk of various cancers (11). In the 1950s, Boyland et al reported for the first time on the preventive effect of the β-glucuronidase inhibitor d-glucaro-1,4-lactone on bladder carcinogenesis (12). It is widely accepted that β-glucuronidase inhibitors, such as d-glucaro-1,4-lactone or its precursors, exert a preventive action on breast, prostate, colon, lung and skin...
Chemicals

Materials and methods

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formation. Then, we demonstrated the inhibition of colon carcinogenesis. A short-term assay was first conducted to
determine whether ACE might inhibit aberrant crypt foci
during tumor initiation by increasing detoxification is therefore
a possible mechanism for cancer prevention (22). On the
other hand, so is the suppression of cell proliferation and the
induction of differentiation and apoptosis during the promotion
and progression stages (23). Proliferating cell nuclear antigen
(PCNA) is a co-factor for DNA polymerase δ that participates
in DNA synthesis and repair. The PCNA assay used in the
present study is a popular method for measuring cell prolif-
eration. It is based on the labeling and then counting of the
labeled cells during the late G1, S, and early G2 phases (24,25).

In this study, we investigated the chemopreventive effects
of ACE and α-glucurono-γ-lactone (GL), synthetic and natural
precursors of α-glucaro-1,4-lactone, respectively, on colon carcinogenesis. A short-term assay was first conducted to
determine whether ACE might inhibit aberrant crypt foci formation. Then, we demonstrated the inhibition of colon
tumorigenesis in rats by ACE and GL in a long-term experi-
ment. Additionally, we investigated whether ACE and GL reduce the rate of cellular proliferation.

Determination of aberrant crypt foci. In the short-term experiment, colons were longitudinally cut open along the
main axis and washed with saline. They were placed between
filter papers and fixed by 10% buffered formalin for at least
24 h. After slides had been stained with a 0.2% methylene blue solution for 30 sec and briefly washed with distilled water,
ACF were identified by their larger size and wider pericryptal
zones using a light microscope at x40 magnification. The
number of ACF per colon, number of aberrant crypts per
section of the crypts was counted

Materials and methods

Immunohistochemistry for cell proliferation (PCNA assay). In

order to measure cell proliferation in the epithelium, PCNA
assays were carried out on the colonic mucosae of 8 rats from
each group. Immunohistochemical staining was performed as
previously described (27,28). The embedded tissues were cut
into 4-μm sections and then stained using anti-PCNA antibo-
dies and the LSAB Kit (Dako, Carpinteria, CA). The number
of PCNA-positive nuclei per section of the crypts was counted
as previously described (27,28), then divided by the total
number of nuclei to determine the PCNA-positive index (%).

A.

B.

Figure 1. Molecular structure of α-glucurono-γ-lactone (A) and aceglatone (B).
Statistical analysis. All data were presented as the means ± SD. Fisher's exact probability test, the Student's t-test and Welch's t-test were used for statistical analysis. A value of p<0.05 was considered significant.

Results

Effect of aceglatone on aberrant crypt foci formation. As shown in Table I, the mean BW of rats treated with 2% ACE was significantly lower than in the Group 1 rats, which were administered AOM alone (p<0.01). However, there were no significant differences in the mean liver weight (LW) or mean relative liver weight (RLW; g/100 g BW) of the rats. The number of ACF/colon in Group 1 (AOM alone) was 133±33.1. Dietary administration of 0.5 or 2% ACE led to a significant reduction in the number of ACF/colon (p<0.01), as well as in the number of aberrant crypts/colon (p<0.01) and aberrant crypts/focus (0.5% ACE, p=0.014; 2% ACE, p<0.01). It also reduced the number of large ACF containing 4 or more aberrant crypts (p<0.05).

Summary of body weight, liver weight, and relative ratio of liver to body weight. No statistically significant difference in dietary intake with or without chemicals (on average 15 g/rat/day) was observed. As shown in Table II, the BW and LW of rats treated with AOM was lower than in the controls, but the
relative ratios between groups were not significantly different. Among the groups treated with AOM, the BW of rats in Group 2 was lower than in Group 1 (p<0.01); LW and RLW did not differ significantly. Histologically, there were no pathologic changes in the liver or kidney of the majority of rats; only one, treated with GL in Group 6, had a liver tumor. Incidence and multiplicity of colonic neoplasms. Most tumors had formed in the large intestine; 2/3 of these were mainly located in the middle and distal colon. Tumors were determined to be sessile or pedunculated, and were histologically diagnosed as adenomas, tubular adenocarcinomas or mucinous carcinomas. Tubular adenocarcinomas had the highest incidence. The incidence and multiplicity of intestinal neoplasms are shown in Table III. AOM alone (Group 1) induced a 37% incidence of large intestinal tumors with a multiplicity of 0.48±0.7. The incidence of large intestinal tumors and total incidence of colon and small intestinal tumors in Groups 2, 3, 4 and 6 were lower than in Group 1. Statistically, the incidence of large intestinal tumors in Groups 2, 3 and 6 was significantly lower than in Group 1 (p<0.05), as was the total incidence of colon and small intestinal tumors in Groups 2 and 6 (p<0.05). As well, the multiplicity of large intestinal tumors and the total multiplicity of colon and small intestinal tumors in Groups 2, 3, 4 and 6 were reduced when compared with Group 1. Statistically, the multiplicity of large intestinal tumors in Groups 2 and 6 was significantly lower (p<0.01 and p<0.05, respectively) than in Group 1. The total multiplicity of colon and small intestinal tumors in Groups 2 and 6 was significantly lower than in Group 1 (p<0.05).

### Table II. Summary of body weight and relative ratio of liver to body weight in the long-term experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>BW (g)</th>
<th>LW (g)</th>
<th>RLW (g/100 g BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>27</td>
<td>350±2±2</td>
<td>13.1±2.5</td>
<td>3.7±0.7</td>
</tr>
<tr>
<td>2</td>
<td>AOM + 2.0% ACE</td>
<td>26</td>
<td>333±17±4</td>
<td>13.4±1.8</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>3</td>
<td>AOM + 0.5% ACE</td>
<td>25</td>
<td>339±22±6</td>
<td>14.2±2.3</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>4</td>
<td>AOM + 0.5% GL</td>
<td>24</td>
<td>360±21±4</td>
<td>14.3±1.9</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>5</td>
<td>AOM + 0.5% GL</td>
<td>26</td>
<td>349±15±5</td>
<td>13.7±1.6</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>6</td>
<td>AOM + 2.0% GL</td>
<td>25</td>
<td>342±15±5</td>
<td>13.3±1.6</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>7</td>
<td>AOM + 0.5% GL</td>
<td>23</td>
<td>347±20±5</td>
<td>13.7±1.7</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>8</td>
<td>Untreated control</td>
<td>6</td>
<td>377±12±2</td>
<td>15.0±1.1</td>
<td>4.0±0.3</td>
</tr>
</tbody>
</table>

aData are the means ± SD. Significantly different from corresponding Group 6 according to *Welch's t-test (p<0.01); Student's t-test (p<0.01); *Welch's t-test (p<0.05); *Student's t-test (p<0.05). Significantly different from corresponding Group 1 according to the Student's t-test (p<0.01). BW, body weight; LW, liver weight; RLW, relative liver weight; AOM, azoxymethane; ACE, aceglatone; GL, ß-glucuronidase inhibitors on colon carcinogenesis.

### Table III. Incidence and multiplicity of intestinal neoplasms in the long-term experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Small intestine</th>
<th>Colon</th>
<th>Total</th>
<th>Incidence (%)</th>
<th>Multiplicitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>27</td>
<td>3 (11)</td>
<td>10 (37)</td>
<td>13 (44)</td>
<td>0.11±0.32</td>
<td>0.48±0.70</td>
</tr>
<tr>
<td>2</td>
<td>AOM + 2.0% ACE</td>
<td>26</td>
<td>3 (12)</td>
<td>2 (8)</td>
<td>5 (19)</td>
<td>0.12±0.33</td>
<td>0.08±0.27</td>
</tr>
<tr>
<td>3</td>
<td>AOM + 0.5% ACE</td>
<td>25</td>
<td>2 (8)</td>
<td>3 (12)</td>
<td>5 (20)</td>
<td>0.08±0.28</td>
<td>0.16±0.47</td>
</tr>
<tr>
<td>4</td>
<td>AOM + 2.0% GL</td>
<td>24</td>
<td>1 (4)</td>
<td>8 (33)</td>
<td>9 (38)</td>
<td>0.04±0.21</td>
<td>0.38±0.58</td>
</tr>
<tr>
<td>5</td>
<td>AOM + 0.5% GL</td>
<td>26</td>
<td>5 (19)</td>
<td>11 (42)</td>
<td>16 (54)</td>
<td>0.22±0.51</td>
<td>0.44±0.58</td>
</tr>
<tr>
<td>6</td>
<td>AOM + 0.5% GL</td>
<td>25</td>
<td>1 (4)</td>
<td>3 (12)</td>
<td>4 (16)</td>
<td>0.08±0.28</td>
<td>0.12±0.33</td>
</tr>
<tr>
<td>7</td>
<td>AOM + 0.5% GL</td>
<td>23</td>
<td>1 (4)</td>
<td>11 (48)</td>
<td>12 (52)</td>
<td>0.04±0.21</td>
<td>0.57±0.73</td>
</tr>
<tr>
<td>8</td>
<td>Untreated control</td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aData are shown as the mean ± SD. Significantly different from Group 1 according to *Fisher's exact probability test (p<0.05); *Welch's t-test (p<0.05); *Welch's t-test (p<0.01). AOM, azoxymethane; ACE, aceglatone; GL, ß-glucuronidase inhibitors on colon carcinogenesis.
The marked inhibitory effects of ACE and of a high dose of GL (2%) observed during the post-initiation phase rather than in the initiation phase of AOM-induced colon carcinogenesis indicate that other mechanisms, in addition to detoxification, might be involved. Increased cellular proliferation is thought to play an important role in multi-stage carcinogenesis (33). In the present study, we evaluated the PCNA-positive cell index in colonic mucosa and found it reduced in the groups treated with ACE and GL. Yoshimi et al have demonstrated that various β-glucuronidase inhibitors have antiproliferative effects in mammary gland, colon and skin carcinogenesis in vivo (34; Walaszek et al, Proc Am Assoc Cancer Res 31: abs. 735, 1990), and that they also inhibit the growth of breast cancer cells in vitro (35). In addition, we recently demonstrated in vitro that ACE has the potential to reduce cell proliferation, induce apoptosis and inhibit DNA synthesis in human colon cancer cells (COLO 320) (Yoshimi et al, unpublished data). Thus, recent studies suggest that the chemopreventive effects of ACE and GL could be explained, in part, by their ability to inhibit the increased proliferation of colonic epithelium cells induced by AOM treatment, as well as by their ability to induce apoptosis.

In conclusion, the results of the present study demonstrate the inhibitory effects of ACE and GL on the initiation and post-initiation phases of AOM-induced colon carcinogenesis in rats. As precursors of the β-glucuronidase inhibitor D-glucaro-1,4-lactone, ACE and GL might exert their chemopreventive action through multiple mechanisms, including the inhibition of β-glucuronidase, the reduction of cell proliferation and the induction of apoptosis. Pre-clinical efficacy and other possible mechanisms thus need to be further studied.

Acknowledgements

We wish to thank K. Takahashi for his excellent technical assistance. This work was supported, in part, by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan and NIH grant P30 CA 54174-16S1.

References


