Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure

Michihiro Mutoh 2, Mami Takahashi, Kazunori Fukuda 1, Yuko Matsushima-Hibiya, Hiroshi Mutoh 2, Takashi Sugimura and Keiji Wakabayashi 3

Cancer Prevention Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, 1Department of Oriental Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, Japan and 2Department of Gastroenterology, Institute of Clinical Medicine, Tsukuba University, Tennoudai 1-11, Ibaraki 305-0006, Japan

1To whom correspondence should be addressed
Email: kwakabay@gan2.ncc.go.jp

Cyclooxygenase-2 (COX-2) is abundantly expressed in colon cancer cells. It has been reported that inhibition of COX-2 enzyme activity is shown to prevent colon carcinogenesis. Thus, suppression of COX-2 expression may also be an effective chemopreventive strategy. In the present study, we constructed a β-galactosidase reporter gene system in human colon cancer DLD-1 cells, and measured COX-2 promoter-dependent transcriptional activity in the cells. Interferon γ suppressed this COX-2 promoter activity, while 12-O-tetradecanoylphorbol-13-acetate and transforming growth factor α (TGFα) exerted enhancing effects. We then tested the influence of 14 candidate cancer chemopreventive compounds on COX-2 promoter activity. Chemopreventive agents such as quercetin, kaempferol, genistein, resveratrol and resorcinol, all having a common resorcin moiety, were found to effectively suppress the COX-2 promoter activity with and without TGFα-stimulation in DLD-1 cells. Since all these compounds have a resorcin moiety as a common structure, a resorcin-type structure may play an active role in the inhibition of COX-2 expression in colon cancer cells.

Introduction

Colorectal cancer is currently one of the major causes of death from cancer in developed countries and, thus the search for chemopreventive agents effective in the large bowel has become very important. Many epidemiological and experimental studies have demonstrated that inhibition of cyclooxygenase (COX) is an effective measure in reducing the risk of colon carcinogenesis (1,2). COX catalyzes the oxygenation of arachidonic acid, leading to the formation of prostaglandins. Recently, the presence of two isoforms of COX has been established (3), a constitutive enzyme, COX-1, present in many cells and tissues, and an inducible enzyme, COX-2, observed in cells in response to growth factors, mitogens and pro-inflammatory cytokines (3,4). COX-1 is constitutively expressed and has a ‘housekeeping’ role helping to maintain physiological functions such as cytoprotection and blood flow.

In contrast, COX-2 is not present under normal physiological conditions but is upregulated with inflammation and colorectal tumor formation (5–7). Recent studies have suggested that overexpression of COX-2 and the resultant overproduction of prostaglandins might be involved in the development of colon cancer (8).

It has been demonstrated that COX-2 selective inhibitors suppress spontaneous and chemically induced intestinal tumor formation in animal experiments (9–11). It has been also reported that inactivation of the COX-2 gene in the ApC knockout mouse, a model of human familial adenomatous polyposis, or treatment of these mice with COX-2 selective inhibitors, dramatically reduces the size and number of intestinal polyps (12). It is thus likely that increased expression of COX-2 is an important contributor to colon tumor formation, and compounds that inhibit the activity and/or expression level of this enzyme are potentially of great interest as candidate chemopreventive agents against colon carcinogenesis. Although major efforts have been made to develop selective inhibitors of COX-2 as chemopreventive agents against colon cancer, efforts to identify agents that can selectively suppress the expression of COX-2 at the gene level appear to be equally important. It is also likely that the combination of suppression of COX-2 gene expression and selective inhibition of its enzyme activity may provide the most effective approach to colon cancer prevention. Therefore, developing a simple screening system, which could detect the suppression of COX-2 gene expression, might be useful for searching novel chemopreventive agents.

In the present study, we used a β-galactosidase (β-gal) reporter gene system to estimate COX-2 promoter activity in human colon cancer cells. The human colon cancer cell line DLD-1 can be stably transfected with a construct harboring the promoter sequence of the COX-2 gene fused to the β-gal reporter gene. Using this β-gal reporter gene system, we evaluated the effects of various cytokines and a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) on COX-2 promoter activity, and also tested various potential chemopreventive agents for their effects on COX-2 promoter activity.

Materials and methods

Chemicals

Ascorbic acid, alpha-tocopherol, curcumin, epigallocatechin gallate, resveratrol, tannic acid, transforming growth factor α (TGFα) and TPA were obtained from Sigma Chemical Co. (St Louis, MO). Beta-carotene, daidzein and kaempferol were from Extrasynthese (Genay, France). Genistin was purchased from Fujicco Co. (Kobe, Japan). Genistein, glutathione (reduced form), quercetin and resorcinol were from Wako Pure Chemical Industries (Osaka, Japan). Docosahexaenoic acid (DHA)-ethyl ester was obtained from Sagami Chemical Research Center (Sagamihara, Japan) and interleukin 1β (IL-1β) and interferon γ (IFNγ) from Genzyme (Cambridge, MA).

Cell culture

DLD-1 cells, a human colon adenocarcinoma cell line, were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and antibiotics (100 μg/
ml streptomycin and 100 U/ml penicillin) at 37°C in 5% CO₂. Cells (2.0 × 10⁵ cells/ml) were plated in 96-well tissue culture plates and preincubated for 24 h before treatment with test agents.

**MTT assay**

Cell viability in each culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatment, the cells were further incubated in a medium containing 0.5 mg/ml of MTT for 1 h. The MTT formazan produced by living cells was dissolved in dimethyl sulfoxide and absorbance at 595 nm was measured on a microplate Reader (Bio-Rad Laboratories, Hercules, CA).

**Reporter gene assay for COX-2 promoter-dependent transcriptional activity**

Human genome DNA was isolated from peripheral lymphocytes obtained from a healthy volunteer. A 2078 nucleotide human COX-2 gene promoter fragment stretching from −2046 to +32 (relative to the transcription start site), containing all known transcription factor responsive elements and the transcription start site of the gene (13), was amplified by PCR using a commercial PCR kit (Takara Shuzo Co., Otsu, Japan). PCR primers corresponding to sequences at the 5' end (forward primer, 5'-GGAACT-CTTCACCTCTATCTGGTATAAGGTA-3') and the 3' end (anti-sense primer, 5'-TCTCTGACGCTACCTGCAAATGTAATGACATT-3') of the 5'-flanking region, from −2046 to +32, of the human COX-2 gene were synthesized by Sawady Technology Co. (Tokyo, Japan). The DNA fragment was inserted into the upstream site of the lacZ gene of the pβ2-βGal-BSD vector, which contains the lacZ gene and blasticidin-S deaminase (BSD) gene. The resultant fusion plasmid was named pCOX2/B2-βGal-BSD.

DLD-1 cells were transfected either with pB2-βGal-BSD or pCOX2/B2-βGal-BSD plasmid DNA. For transfection, 2 × 10⁵ DLD-1 cells were grown in 10 cm culture dishes. After overnight adhesion, cells were transfected with 15 μl of plasmid DNA using Tfx™-20 (Promega, Madison, WI) according to the instructions provided by the manufacturer. Stable transfectants were selected in a medium containing 20 μg/ml blasticidin-S hydrochloride (Kaken Pharmaceutical Co., Tokyo, Japan). Stable transfectants containing pB2-βGal-BSD in the genome DNA of DLD-1 cells were designated as DLD-1/B2-βGal-BSD, and those containing pCOX2/B2-βGal-BSDwere designated as DLD-1/COX2-B2-βGal-BSD. From heterogeneous populations of DLD-1/COX2-B2-βGal-BSD cells, we obtained a subclone that contained an intact DNA fragment of 2078 bp COX-2 promoter region and downstream lacZ gene in the genome DNA, by the limiting dilution method and used it for the present study. The presence of the COX-2 gene promoter was confirmed by the size of the PCR product and also by DNA sequencing. The subcloned DLD-1/COX2-B2-βGal-BSD cells were seeded at a density of 2 × 10⁵ cells/well in 96-well microtiter plates and preincubated for 24 h. The cells were then treated with test agents, and β-gal activity of DLD-1 cells in each well was determined by a colorimetric assay using o-nitrophenyl-β-galactopyranoside. The background β-gal activity was determined in control untreated culture of DLD-1/B2-βGal-BSD cells, and the value set as 100%. The percent β-gal activity with each treatment was calculated from data for triplicate wells. The value was normalized for viable cell number, assessed by the MTT assay. All experiments were repeated at least three times with nearly identical results. Data are expressed as means ± SE (n = 3).

**Results**

**Effect of cytokines and TPA on COX-2 promoter activity**

No significant difference was observed in the basal COX-2 promoter activity of DLD-1/COX2-B2-βGal-BSD cells between serum-deprived cultures and 5% FBS-supplemented cultures after 48 h. To examine if the reporter gene assay was working correctly, cells were cultured in a medium containing 5% FBS, and the effects of TPA, IL-1β, TGFα and IFNγ on COX-2 promoter activity were tested. Treatment of cells with 200 ng/ml TPA, 10 ng/ml IL-1β or 100 ng/ml TGFα for 48 h increased the activity to 1.8, 1.3 and 2.1 times the control value, respectively (Figure 1A). Treatment with 10 ng/ml IFNγ resulted in an 85% decrease of COX-2 promoter activity (Figure 1A). Dose–response studies showed that IFNγ at 0.02–4.0 ng/ml suppressed COX-2 promoter activity in a dose-dependent manner (Figure 1B). No significant decrease of cell viability was observed after 48 h culture with IFNγ at these concentrations.

---

**Table I. Compounds tested in this study**

<table>
<thead>
<tr>
<th>Compounda</th>
<th>Final dose (μM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenol</td>
<td>500</td>
</tr>
<tr>
<td>Daiztein</td>
<td>500</td>
</tr>
<tr>
<td>Epigallocatechin gallatec</td>
<td>500</td>
</tr>
<tr>
<td>Genestinc</td>
<td>40</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>40</td>
</tr>
<tr>
<td>Quercetinc</td>
<td>20</td>
</tr>
<tr>
<td>Resorcinolb</td>
<td>500</td>
</tr>
<tr>
<td>Resveratrolb</td>
<td>100</td>
</tr>
<tr>
<td>Vitaminsc</td>
<td></td>
</tr>
<tr>
<td>Alpha-tocopherol</td>
<td>100</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100</td>
</tr>
<tr>
<td>Beta-carotene</td>
<td>100</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>100</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>10</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>10</td>
</tr>
<tr>
<td>Glutathione (reduced form)</td>
<td>5000</td>
</tr>
</tbody>
</table>

aThe underlined samples suppressed COX-2 promoter activity.
bFinal dose tested in this study.
cCompounds have a resorcin-type moiety as a common structure.

**Effects of chemopreventive agents on COX-2 promoter activity**

The effects of the 14 compounds listed in Table I on TGFα-stimulated COX-2 promoter activity were examined. These compounds are known to have cancer chemopreventive properties and also have anti-oxidative properties. Among those, quercetin, kaempferol, genistein, resveratrol and resorcinol significantly suppressed TGFα-stimulated COX-2 promoter activity in a dose-dependent manner, respectively (Figure 2). Decreases in TGFα-stimulated COX-2 promoter activity at the highest doses of test samples were as follows; 74% for 20 μM quercetin, 49% for 40 μM kaempferol, 79% for 40 μM genistein, 81% for 100 μM resveratrol and 53% for 500 μM resorcinol (Figure 2A–E). Thus, quercetin, genistein and resveratrol suppressed the TGFα-stimulated COX-2 promoter activity to below the basal level, but kaempferol and resorcinol did not. No response was seen in nine compounds listed in Table I. For example, daidzein did not show any activity, even at the dose of 500 μM.

Next, the effects of quercetin, kaempferol, genistein, resveratrol and resorcinol on COX-2 promoter activity in DLD-1 cells in the absence of TGFα were examined. These five compounds
Suppression of COX-2 by chemopreventive agents

Fig. 4. Chemical structures of quercetin, kaempferol, genistein, resveratrol, resorcinol and daidzein. All agents have a common resorcin moiety, except for daidzein, indicated by the box.

Different effects were shown by quercetin, genistein and resveratrol. No significant decrease in cell viability was observed after 48 h culture with quercetin, kaempferol, genistein and resorcinol at the doses used in the experiments. However, resveratrol (72% cell viability in 25 µM concentration) reduced cell viability. Other compounds showed no significant suppressive effects on COX-2 promoter activity when used at the concentrations listed in Table I, and these doses gave ~60% cell viability.

Fig. 2. Effects of chemopreventive agents on COX-2 promoter activity in TGFα-treated DLD-1 cells. DLD-1/COX-2-B2-βgal-BSD cells were cultured in the presence of 100 ng/ml TGFα with quercetin (A), kaempferol (B), genistein (C), resveratrol (D), resorcinol (E) and daidzein (F) at the indicated concentrations. COX-2 promoter activity was normalized for viable cell numbers assessed by MTT assay and plotted as the percentage of the unstimulated control culture value. Data are means ± SE (n = 3). Similar results were obtained from three separate experiments.

Fig. 3. Suppression of the level of COX-2 promoter activity in unstimulated DLD-1 cells by chemopreventive agents. DLD-1/COX-2-B2-βgal-BSD cells were cultured in medium containing 5% FBS without addition of TGFα. Cells were cultured for 48 h in the presence of quercetin (A), kaempferol (B), genistein (C), resveratrol (D) and resorcinol (E) at the indicated concentrations. COX-2 promoter activity was shown according to the procedures described in Figure 2.

Discussion
Since COX-2 is an interesting target for cancer chemoprevention, a reporter gene assay of COX-2 promoter activity in the human colon cancer cell line DLD-1 was used for screening of its suppressors in the present study. COX-2 promoter activity was detected in DLD-1 cells without stimulation, and this was clearly enhanced by TPA and TGFα, but suppressed by IFNγ, which was previously reported to reduce levels of COX-2 mRNA in airway epithelial cells (14). Paradoxically, IFNγ is also known to be a strong stimulator of COX-2 expression in macrophages (15).

Using our reporter gene assay system, testing of 14 natural compounds known to have cancer chemopreventive activity showed quercetin, kaempferol, genistein, resveratrol and resorcinol to suppress COX-2 expression in macrophages (18), endothelial cells (19) and fibroblasts (20). Resveratrol has also been demonstrated to suppress COX-2 expression in mammary epithelial cells (21). Thus, our results in colon cancer cells are in agreement with those of early studies. The above five inhibitors of COX-2 promoter activity are known to be antioxidants and are widely distributed in various foodstuffs including fruit and vegetables (22–28). Quercetin, kaempferol, genistein and resveratrol have also been reported to inhibit tyrosine kinases (29–31), which are involved in the induction suppressed the COX-2 promoter activity in colon cancer cells in a dose-dependent manner, as shown in Figure 3A–E. Treatment with 20 µM quercetin, 40 µM kaempferol, 100 µM resveratrol, 40 µM genistein or 500 µM resorcinol resulted in 42, 47, 66, 58 and 49% decreases in COX-2 promoter activity, respectively. Nearly the same inhibitory effect of the COX-2 promoter activity with and without TGFα stimulation was shown by kaempferol and resorcinol. On the other hand,
of COX-2 expression (19) and known to play important roles in cell proliferation and transformation (32). It has been well established that TPA and TGFGz activate protein-tyrosine kinases (PTKs) (33,34). TGFGz binds to the epidermal growth factor receptor, one of the receptor PTKs, and signals from activated PTKs are transduced to downstream signaling pathways. The main signaling pathway is the Ras/ERK-MAPK pathway, and others are the JAK-STAT pathway, the PI3 kinase-Akt pathway, the P38 pathway, etc. (32). Ras/ERK-MAPK, JAK-STAT and P38 pathways are known to be involved in the induction of gene expression of COX-2 (35–37). Therefore, one of the possible mechanisms by which the five agents suppressed the TGFGz-mediated activation of COX-2 transcription in the present study is considered to be inhibition of the activation of PTKs. LDL-1 cells have an activating mutation in the K-ras gene (38) and also produce TGFGz protein (39). These might contribute to the basal expression of COX-2. Thus, the basal level of COX-2 promoter activity might also be suppressed by inhibiting the activation of PTKs. Kaempferol and resorcinol suppressed both TGFGz-stimulated and non-stimulated COX-2 promoter activity to nearly the same degree, and quercetin, genistein and resveratrol showed different effects. These two situations might be the result of inhibiting different signaling pathways. Further examination is needed to identify the inhibiting pathways.

As shown in Figure 4, quercetin, kaempferol, genistein and resveratrol, which exhibited inhibitory effects on COX-2 promoter activity, have a common resorcin moiety in their structure. Daidzein, an analog of genistein, but not having a resorcin moiety in its structure and an inhibitory activity of PTKs, did not inhibit COX-2 promoter activity. Moreover, resorcinol itself suppressed COX-2 promoter activity. Therefore, the resorcin moiety may play a critical role in the inhibition of COX-2 promoter activity, although other physicochemical factors may also be involved. On the other hand, epigallocatechin gallate showed no inhibitory effect on COX-2 promoter activity, even though its structure contains a resorcin moiety and it inhibits PTKs activities. Elements other than a resorcin moiety in the structure of epigallocatechin gallate may be involved in the absence of the inhibition of COX-2 promoter activity. Further examination is needed to determine the relationship between the structures of polyphenols and the inhibition of COX-2 promoter activity. Moreover, studies are required to show whether compounds exhibiting in vitro activity suppress COX-2 expression in vivo.

In conclusion, our method has potential in the search for novel inhibitors of COX-2 expression and such compounds might be effective as anti-inflammatory and/or cancer preventive agents. Suppression of COX-2 expression by quercetin, kaempferol, genistein, resveratrol and resorcinol could be involved in their mechanisms of cancer prevention.

Acknowledgements

This work was supported in part by a grant from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan, a Grant-in-Aid from the Ministry of Health and Welfare for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, and a grant from TAKEDA Science Foundation. M.M. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

References


Received August 6, 1999; revised January 31, 2000; accepted February 2, 2000