Downregulation of prostaglandin E receptor subtype EP\textsubscript{3} during colon cancer development

Y Shoji, M Takahashi, T Kitamura, K Watanabe, T Kawamori, T Maruyama, Y Sugimoto, M Negishi, S Narumiya, T Sugimura, K Wakabayashi

Background and aims: Involvement of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) receptors EP\textsubscript{1}, EP\textsubscript{2}, and EP\textsubscript{4} in the formation of aberrant crypt foci (ACF) and/or intestinal polyps has been suggested. In contrast, EP\textsubscript{3} appears to have no influence on the early stages of colon carcinogenesis. In the present study, we examined expression of PGE\textsubscript{2} receptor subtypes EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} in normal colon mucosa and colon cancers, and assessed the contribution of EP\textsubscript{3} to colon cancer development.

Methods: mRNA expression of PGE\textsubscript{2} receptor subtypes EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} was compared in normal colon mucosa and colon cancers in azoxymethane (AOM) treated mice and rats, and in humans, were examined by reverse transcription-polymerase chain reaction (RT-PCR), quantitative real time RT-PCR, and immunohistochemical analyses. Evaluation of the role of EP\textsubscript{3} was performed by intraperitoneal injection of AOM, using EP\textsubscript{3} receptor knockout mice. Effects of EP\textsubscript{3} receptor activation on cell growth of human colon cancer cell lines were examined using ONO-AE-248, an EP\textsubscript{3} selective agonist. Moreover, EP\textsubscript{3} expression in colon cancer cell lines was analysed with or without 5-aza-2'-deoxycytidine (5-aza-dC) treatment.

Results: Expression levels of EP\textsubscript{1} and EP\textsubscript{2} mRNA were increased in cancer tissues. EP\textsubscript{3} mRNA was constantly expressed in normal mucosa and cancers. In contrast, expression of EP\textsubscript{3} mRNA was markedly decreased in colon cancer tissues, being 5% in mice, 9% in rats, and 28% in humans compared with normal colon mucosa, analysed by quantitative real time RT-PCR. Immunohistochemical staining demonstrated the rat EP\textsubscript{3} receptor protein to be expressed in epithelial cells of normal mucosa and some parts of small carcinomas but hardly detectable in large carcinomas of the colon. Colon cancer development induced by AOM in EP\textsubscript{3} receptor knockout mice was enhanced compared with wild-type mice, with a higher incidence of colon tumours (78% v 57%) and mean number of tumours per mouse (2.17 (0.51) v 0.75 (0.15); p<0.05). Expression of EP\textsubscript{3} mRNA was detected in only one of 11 human colon cancer cell lines tested. Treatment with 5 \mu M of an EP\textsubscript{3} selective agonist, ONO-AE-248, resulted in a 30% decrease in viable cell numbers in the HCA-7 human colon cancer cell line in which EP\textsubscript{3} was expressed. Treatment with 5-aza-dC restored EP\textsubscript{3} expression in CACO-2, CW-2, and DLD-1 cells but not in WiDr cells, suggesting involvement of hypermethylation in the downregulation of EP\textsubscript{3} to some extent.

Conclusion: The PGE\textsubscript{2} receptor subtype EP\textsubscript{3} plays an important role in suppression of cell growth and its downregulation enhances colon carcinogenesis at a later stage. Hypermethylation of the EP\textsubscript{3} receptor gene could occur and may contribute towards downregulating EP\textsubscript{3} expression to some extent in colon cancers.

Clear benefits have been reported in epidemiological studies with non-steroidal anti-inflammatory drugs (NSAIDs) as chemopreventive agents against colon cancers, one of the most common malignancies in rodents.\textsuperscript{1} Chemically induced colon carcinogenesis in rodents is also suppressed by administration of NSAIDs.\textsuperscript{2,4} Moreover, intestinal polyp formation in familial adenomatous polyposis coli patients is markedly reduced after application of agents such as sulindac or indomethacin.\textsuperscript{1,4} The common mechanism of action of NSAIDs is inhibition of cyclooxygenase (COX) activity, two distinct isoforms of which have been reported: a constitutive enzyme, COX-1, and an inducible enzyme, COX-2.\textsuperscript{6} COX-1 and COX-2 are rate limiting enzymes in the synthesis of prostanoids which affect cell proliferation, tumour growth, apoptosis, and immune responsiveness, and both COX isoforms have been reported to be involved in colon carcinogenesis.\textsuperscript{1,4} 10 Prostanoids such as prostaglandin (PG)E\textsubscript{2}, PGD\textsubscript{2}, PGE\textsubscript{2}F\textsubscript{2α}, PGI\textsubscript{2}, and TXA\textsubscript{2} exert their biological actions through binding to nine specific receptors with seven transmembrane domains: the four subtypes EP\textsubscript{1}–EP\textsubscript{4} for PGE\textsubscript{2}, DP and CRTH2 for PGD\textsubscript{2}, FP for PGE\textsubscript{2}F\textsubscript{2α}, IP for PGI\textsubscript{2}, and TP for TXA\textsubscript{2}.\textsuperscript{11,12} Several reports have demonstrated increased levels of PGE\textsubscript{2} in human colon cancer tissues compared with surrounding normal mucosa.\textsuperscript{13-5} Signal transduction pathways of PGE\textsubscript{2} receptors have been studied by examining agonist induced changes in the levels of second messengers such as cAMP and free Ca\textsuperscript{2+} and by identifying G protein coupling by various methods.\textsuperscript{15} The EP\textsubscript{1} receptor is known to mediate PGE\textsubscript{2} induced elevation of free Ca\textsuperscript{2+} concentration although the species of G protein to which EP\textsubscript{1} receptor is coupled remains unidentified. EP\textsubscript{2} and EP\textsubscript{4} receptors are coupled to Gs and stimulate cAMP production by adenylate cyclase. In contrast, the major signalling pathway for the EP\textsubscript{3} receptor is inhibition of adenylate cyclase via Gi. In addition, another function has been suggested for this receptor type in which cell phenotype is regulated through activation of Rho via G proteins other than Gi.\textsuperscript{16}
Establishment of mice lacking the genes encoding prosta
to receptors has promoted understanding of the involve-
ment of prostanoids in the development of colon cancer. In previous studies, we demonstrated that deficiency of either EP1 or EP2 receptor decreases formation of azoxymethane (AOM) induced aberrant crypt foci (ACF), putative preneoplastic lesions in the colon. Moreover, antagonists of EP3 and EP4 receptors suppress formation of AOM induced ACF in the colon of mice and intestinal polyp formation in Apc gene deficient Min mice. Recently, it was also reported that homogenous deletion of the gene encoding the EP3 receptor resulted in a decrease in intestinal polyp formation in Apc knockout mice. As already mentioned, EP3 and EP4 stimulate adenylate cyclase whereas EP2 exerts an inhibitory influence, suggesting a possible suppressive role against colon carcinogenesis. However, deficiency of EP2 did not affect AOM induced ACF formation in our previous study.

In the present study, we hypothesised that EP3 might act at a later stage in colon carcinogenesis. Examination of mRNA expression for EP1, EP2, EP3, and EP4 in colon carcinomas of mice, rats, and humans demonstrated that levels of EP3 were markedly decreased compared with normal mucosa. An increase in colon carcinoma formation induced by AOM was also demonstrated in EP3 receptor knockout mice. Furthermore, activation of the EP3 receptor showed a suppressive effect on cell growth in a colon cancer cell line in which EP3 was expressed. In most human colon cancer cell lines tested, EP3 expression was not detected but treatment with 5-azacytidine (5-aza-dC) restored EP3 expression in some cell lines. On the basis of the results obtained, the role of the EP3 receptor in colon carcinogenesis is discussed.

MATERIALS AND METHODS

Animals

The mouse gene encoding the PG_E receptor EP3 was disrupted by a gene knockout method using homologous recombination, as reported previously. The generated chimeric mice were backcrossed with C57BL/6Cr mice, and the resulting homozygous mutant mice of these F2 progeny were used at 6 weeks of age. Genotypes of the knockout mice were determined by PCR analysis. The number of molecules of specific gene products in each sample was determined using a standard curve analysis. The number of molecules of specific gene products in each sample was determined using a standard curve analysis.

Colon tumour samples and cell lines

Mouse colon tumours and normal colon mucosa tissues were obtained from C57BL/6Cr male mice treated with AOM, as previously reported. Rat colon tumours and normal colon mucosa tissues were obtained from eight F344 male rats treated with AOM, as previously reported. Frozen samples of mouse and rat tissues were used for reverse transcription (RT)-PCR analyses, and formalin fixed, paraffin embedded rat tissue samples were employed for immunohistochemical staining.

Surgical specimens of human colon cancer and adjacent normal colon mucosa tissues were taken from eight Japanese patients who had undergone surgical operations for colorectal cancers at the National Cancer Center Hospital, Tokyo, and samples were immediately frozen in liquid nitrogen.

Eleven human colon cancer cell lines were subjected to RT-PCR analysis. HCA-7 colony 29, a human colon adenocari- noma cell line, was kindly provided by Dr Susan Kirkland, Imperial College of Science, Technology, and Medicine (London, UK). HCA-7 cells were maintained in Dulbecco’s minimum essential medium supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah, USA) and antibiotics (100 μg/ml of streptomycin and 100 units/ml of penicillin) at 37°C in 5% CO2. Colo 201, LBL-1, HCT-116, SW48, SW480, SW620, WiDr (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), CACO-2, Colo 320, and CW-2 (Riken Cell Bank, Tsukuba, Japan) were purchased and cultured according to the manufacturer’s instructions.

Analysis of EP receptor expression in colon cancers by RT-PCR

Total RNA was extracted from tissues and cultured cells by direct homogenisation in Isogen (Nippon Gene Co., Tokyo, Japan), and spectrophotometry was used for quantification. Aliquots (3 μg) of total RNA were subjected to the RT reaction with oligo-dT primer using an Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany). After reverse transcription, PCR was carried out with Hotstartaq (Qiagen), according to the manufacturer’s instructions. To test cDNA integrity, the β-actin gene was amplified for each sample. Primers were designed using the computer program OLIGO 4.0-s (National Biosciences, Maryland, USA) and were based on published sequences in Genbank. Primers were designed to cross an exon-exon boundary or insertion of intron to ensure that genomic DNA was not being amplified. BLAST searches confirmed that the primers were specific for the target gene. Primers for the β-actin and EP receptor genes are listed in table 1. PCR amplifications were performed in a thermocycler (Gene Amp PCR System 9600; Perkin-Elmer Applied Biosystems, Foster City, California, USA), with 18–40 cycles of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for one min using the specific primer sets. PCR products were then analysed by electrophoresis on 2% agarose gel.

Quantitative real time RT-PCR analysis

Quantitative real time RT-PCR analysis was performed using the Smart Cycler system with the Ex Taq R-PCR version 2 kit and SYBR Green (Takara Shuzo Co., Shiga, Japan) according to the manufacturer’s instructions. Primers for the β-actin and EP receptor genes, and cycle conditions for PCR, are listed in table 2. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analysed by their melting point curves and generally run on 2% agarose gels to confirm the correct sizes of the PCR products. Each PCR product was subcloned into the TA cloning plasmid vector pGEN-T easy vector (Promega Co., Madison, Wisconsin, USA) and used as a positive control for real time PCR analyses. The number of molecules of specific gene products in each sample was determined using a standard curve generated by amplification of 105–106 copies of the control plasmid. Each sample was analysed in triplicate.

Immunohistochemical staining

Immunohistochemical analyses of colon tumours and normal mucosa samples from F344 male rats treated with AOM were performed with the avidin-biotin complex immunoperoxidase technique, as previously reported. As the primary antibody, a polyclonal rabbit anti-EP3 antibody raised against rat EP3 receptors was used at a 1:50 dilution. As the secondary antibody, biotinylated antirabbit IgG (H+L) raised in a goat, affinity purified, and absorbed with rat serum (Vector Laboratories, Inc., Burlingame, California, USA) was used at a 200× dilution. Staining was performed using avidin-biotin reagents (Vectastain ABC reagents; Vector Laboratories, Inc.), 3,3'-diaminobenzidine, and hydrogen peroxide, as previously published. Immunoperoxidase activity was visualised as brown stained cell nuclei or cytoplasm.
**Table 1** List of primers used for reverse transcription-polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Source</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (3′→5′)</th>
<th>Product size (bp)</th>
<th>Cycle No</th>
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<tr>
<td>β-Actin</td>
<td>Mu</td>
<td>NM_007393 AACACCCAGAGCTGACG (Exon 4)</td>
<td>CGTCAAGGAGCAGGTA (Exon 6)</td>
<td>623</td>
<td>22</td>
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<tr>
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<td>Rat</td>
<td>NM_031144 AACACCCAGAGCTGACG (Exon 4)</td>
<td>CGTCAAGGAGCAGGTA (Exon 6)</td>
<td>623</td>
<td>18</td>
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<tr>
<td></td>
<td>Hu</td>
<td>NM_001101 AACACCCAGAGCTGACG (Exon 4)</td>
<td>CGTCAAGGAGCAGGTA (Exon 6)</td>
<td>623</td>
<td>21</td>
</tr>
<tr>
<td>EP1</td>
<td>Mu</td>
<td>NM_013641 CACATTGCAAAGCAGGAC (Exon 2)</td>
<td>CAACACCAAAACAAGTACG (Exon 1)</td>
<td>232</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Hu</td>
<td>NM_009595 GCCATCGGTGGGTCGGT (Exon 2)</td>
<td>GCCCTCTGGTGTGTCCTGA (Exon 3)</td>
<td>317</td>
<td>40</td>
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<tr>
<td></td>
<td>EP2</td>
<td>NM_008904 GTATGGAAGAGGAGGCAG (Exon 1)</td>
<td>ACTGCGACAGCTGAGCTTGA (Exon 2)</td>
<td>295</td>
<td>28</td>
</tr>
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<td></td>
<td>Rat</td>
<td>NM_031086 TGCATGGGCTGGCCGCTGC (Exon 1)</td>
<td>CACACCAAAACAAGTACG (Exon 1)</td>
<td>394</td>
<td>34</td>
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<tr>
<td></td>
<td>Hu</td>
<td>NM_009595 CACCACTCTTTTTGCTG (Exon 1)</td>
<td>CACACCAAAACAAGTACG (Exon 1)</td>
<td>216</td>
<td>34</td>
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<td>EP3</td>
<td>Mu</td>
<td>D10204 TGGTGCCGTGGGTCGGT (Exon 1)</td>
<td>ACTCCTTCTTCTTTCTT (Exon 1)</td>
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<td>Rat</td>
<td>D14869 CTCTTGCTGCTGCTGTG (Exon 1)</td>
<td>CGAAGGCAGGATAGGCA (Exon 2)</td>
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<td>35</td>
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<tr>
<td></td>
<td>Hu</td>
<td>D38297 CTTCCGCTGCTGCTGTG (Exon 1)</td>
<td>CTTCCGCTGCTGCTGTG (Exon 2)</td>
<td>300</td>
<td>35</td>
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<td>EP4</td>
<td>BC011193 CTTGCTGCTCCCTGCTCT (Exon 1)</td>
<td>AAACACCCAGAGCTGACG (Exon 2)</td>
<td>216</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Hu</td>
<td>NM_032076 GCTCAGCTGACTTCTGCG (Exon 1)</td>
<td>GCTCTGCTGACAGCTG (Exon 2)</td>
<td>326</td>
<td>35</td>
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<td>R</td>
<td>NM_009598 TGGTATGCTGGTGCTG (Exon 2)</td>
<td>GAGAGCTGCTGAGCAGA (Exon 3)</td>
<td>329</td>
<td>35</td>
</tr>
</tbody>
</table>

*Mu, mouse; Hu, human.*

5′-Azacytidine treatment
Caco-2, CW-2, DLD-1, HCA-7, and WiDr cells were seeded at a density of $5 \times 10^4$ cells/10 cm dish on day 0 and treated with 1 and 2 μM 5-aza-2′-deoxycytidine (Sigma, St Louis, Missouri, USA) on days 1, 3, and 5. After each treatment, cells were placed in fresh media and harvested on day 6, and total cellular DNA was prepared using Isogen on day 7.

**RESULTS**

Expression of PG2 receptors EP1, EP2, EP3, and EP4 in normal colon mucosa and colon tumours of AOM treated mice and rats, and in human tissues, were examined by RT-PCR (figs 1, 2). In the three mouse colon adenocarcinomas tested, expression of EP1 and EP2 receptor mRNAs was increased compared with levels in normal mucosa. EP4 mRNA was equally expressed in carcinomas and normal mucosa. In contrast, expression of EP3 mRNA was markedly decreased in all carcinoma samples compared with normal colon mucosa (fig 1A). Expression patterns of EP1, EP2, EP3,
and EP3 receptors in eight pairs of samples of adenocarcinoma and normal mucosa from AOM treated rats were similar to those in mice. Patterns for EP1, EP2, EP3, and EP4 receptors in four typical pairs of samples are shown in fig 1B. In the case of human colon tissues, EP3 receptor mRNA was similar to that in mice. Patterns for EP1, EP2, EP3, and EP4 receptors in four typical pairs of samples of adenocarcinomas compared with adjacent normal colon mucosa are shown. (fig 1D), and 28% in humans (fig 2C) of the average value of that in the respective normal colon mucosa.

Expression of EP3 receptor mRNA was significantly downregulated in tumours, being 5% in mice (fig 1C), 9% in rats (fig 1D), and 28% in humans (fig 2C) of the average value of that in the respective normal colon mucosa.

Localisation of EP3 receptor protein in rat colon tumours
Immunohistochemical analysis of paraffin embedded specimens of eight colon tumours and normal colon mucosa in rats treated with AOM was performed. Slight background staining was widely detected in both negative controls, those stained without antirat EP3 receptor antibody (fig 3A, B) and those stained with anti-EP3 receptor antibody preabsorbed with fusion EP3 receptor protein (fig 3C, D). Moreover, slight non-specific staining was detected in red blood cells. In normal colon mucosa tissues, EP3 receptor expression was prominent in epithelial cells (fig 3E), and the muscular coat was also positively stained. Similarly, positive staining of EP3 receptors was observed in hyperplastic ACF of the colon (data not shown). In contrast, staining was very faint, minimal, or absent in epithelial cells of colon adenocarcinomas (fig 3F), being totally lacking in seven cases, sized 3–9 mm in diameter. Only one carcinoma sample was weakly stained, and its size was 2 mm.

Colon tumour development in EP3 receptor knockout mice
To assess the role of EP3 receptors in colon tumour development, EP3 receptor knockout mice were used in an in vivo model. Data for the incidence (percentage of mice with tumours) and multiplicity (number of tumours per
mouse) of colon tumours induced by AOM are summarised in table 3. Tumour incidence was increased to 78% in EP3 receptor knockout mice compared with 57% in wild-type mice. Regarding tumour multiplicity, values were 2.17 (0.51) for EP3 receptor knockout mice and 0.75 (0.15) for wild-type mice (p<0.05). Histopathological examination revealed 20 colon tumours to be adenocarcinomas in wild-type, and 50 colon tumours to be three adenomas and 47 adenocarcinomas in EP3 receptor knockout mice. Figure 4 shows the size distribution, demonstrating a significant increase in tumours measuring >2.0 mm in diameter in EP3 receptor knockout mice (2.00 (0.48) v 0.50 (0.11); p<0.01) but not in those measuring ≤2.0 mm in diameter (0.17 (0.08) v 0.25 (0.11)).

Expressions of PGE2 receptors in colon cancer cell lines, and effects of the EP3 selective agonist on growth of colon cancer cells

Expression of PGE2 receptors in 11 human colon cell lines was examined by RT-PCR. EP1, EP2, and EP4 were widely detected in the human colon cancer cell lines (in 10 of 11 for EP1, nine of 11 for EP2, and nine of 11 for EP4) but EP3 was only detected in HCA-7 (fig 5A).

To evaluate the physiological functions of the EP3 receptor, the effect of an EP3 receptor selective agonist ONO-AE-248 on viable cell numbers of DLD-1 and HCA-7 in monolayer cultures was examined. In the HCA-7 human colon adenocarcinoma cell line, expression of the EP3 receptor and other PGE2 receptors (EP1, EP2, and EP4) were detected by RT-PCR analysis (fig 5A). As shown in fig 5B, HCA-7 cell numbers were significantly decreased dose dependently by addition of ONO-AE-248, with 8%, 17%, and 30% decreases (p<0.05, p<0.01, and p<0.01) in the presence of 1, 3, and 5 μM ONO-AE-248 on day 5, respectively. On the other hand, treatment with ONO-AE-248 did not affect growth of DLD-1 cells which were not expressing EP3 mRNA. The experiments were repeated three times and similar results were obtained.

Effect of 5-aza-dC on EP3 expression

To determine whether silencing by DNA methylation could be involved in reduced expression of EP3 receptor in colon tumours, we tested the effects of 5-aza-dC, a demethylating

### Table 3 Colon tumour development in EP3 receptor knockout mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Incidence</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>16/28 (57%)</td>
<td>0.75 (0.15)</td>
</tr>
<tr>
<td>EP3−/−</td>
<td>18/23 (78%)</td>
<td>2.17 (0.51)</td>
</tr>
</tbody>
</table>

†Number of mice bearing tumours per total number of mice.
‡Number of tumours per mouse. Data are mean (SEM).
*Significantly different from the corresponding wild-type value (p<0.05).
agent, on EP3 receptor expression in colon cancer cell lines. Human colon cancer cell lines CACO-2, CW-2, DLD-1, HCA-7, and WiDr were treated with 5-aza-dC, and expression levels of EP3 receptor were analysed by RT-PCR. Without 5-aza-dC treatment, expression of EP3 receptor was detected in HCA-7, but not in CACO-2, CW-2, DLD-1, or WiDr (fig 5A). After 5-aza-dC treatment, expression was restored in CACO-2, CW-2, and DLD-1, but not in WiDr (fig 6).

**DISCUSSION**

In the present study, examination of mRNA expression levels for EP1, EP2, EP3, and EP4 receptors in colon tissues in mice, rats, and humans by RT-PCR and quantitative RT-PCR provided evidence of a marked reduction in EP3 receptors in colon cancers, in clear contrast with the increase observed for EP1 and EP2. Additionally, results of mRNA expression of EP receptors in 11 human colon cancer cell lines support the above findings and further indicate the events may occur in colon cancer cells. Recently, we reported enhancement of AOM induced colon tumour development using EP3 receptor knockout mice, conducted here in the present study, demonstrated that deficiency of EP1 or EP4 receptor reduced formation of AOM induced ACF while EP3 receptors had no effect, using eight types of EP receptor knockout mice.27 Moreover, the size of the tumours was significantly increased. Thus based on our present and previous results, we suggest that the EP3 receptor does not influence the early stage of colon carcinogenesis, including ACF formation, but its downregulation could be important to cancer development at a later stage.

In our present study, PCR primers of mouse, rat, and human EP3 receptors targeted a common sequence in each species. PCR products would be expected to be derived from the entire range of splice variants (figs 1A–B, 2A, 5A). It is noteworthy that there are three splice variants of the EP3 receptor in mice and rats, and nine in humans, coupled to different G protein signalling pathways.28-33 These variants...
are different in the carboxy terminal tail, and the amino acid sequence has an important role in G protein coupling specificity.10 11 Two of the three variants of the mouse EP₃ receptors are EP₃α and EP₃b, which are coupled to Gₛ and cause inhibition of adenylate cyclase.10 The mouse EP₃ receptor, in contrast, is coupled to Gₛ, in addition to Gₛ, and evokes pertussis toxin insensitive cAMP production.11

Preliminarily, we examined expression of three splice variants of mouse EP₃ receptors by RT-PCR using specific primers for each variant, and found EP₃α to be the major form in mouse normal mucosa (data not shown). These observations support the conclusion that the major splice variants of EP₃ receptors are coupled to Gₛ and stimulate cAMP production by this enzyme. Increased cAMP adenylate cyclase in normal colon mucosa in mice. On the other hand, EP₃ and EP₄ receptors are coupled to Gₛ and stimulate cAMP production by this enzyme. Increased cAMP levels result in activation of cAMP dependent protein kinase (PKA) and transcriptional factors that bind to cAMP responsive elements to transactivate the transcription of specific primary response genes that initiate cell proliferation.12 In our previous study,13 the EP₄ receptor selective agonist ONO-AE-329 was shown to enhance colony formation by the HCA-7 human colon adenocarcinoma cell line. The EP₂ receptor selective agonist ONO-AE-248 was demonstrated to suppress cell growth in HCA-7 in the present study. It has been reported that ONO-AE-248 attenuates the rise in intracellular cAMP induced by forskolin, an activator of adenylate cyclase, in CHO cells transfected with EP₃ receptor.23 Therefore, the EP₃ receptor pathway may play an important role in counteracting the effects of EP₂ and EP₄ receptors, and its downregulation in later stages of colon carcinogenesis may enhance cancer development. Additional studies are needed to investigate interactions between the EP₃ receptor signalling pathway and others linked to EP receptors.

Hypermethylation of CpG islands in promoter regions is known to cause silencing of genes in various human cancers,14 15 and silencing of COX-2 and APC genes by hypermethylation has been reported in human colon cancer.16 17 Although hypermethylation of the prostaglandin receptor gene has not been reported,18 19 DNA sequences in the promoter region and exon 1 of the human EP₃ gene are GC rich (Gembank AL031429). Therefore, in the present study, we examined the effects of demethylation of DNA with 5-aza-dC on EP₃ expression in human colon cancer cell lines. Demethylation of five cell lines by 5-aza-dC treatment resulted in restoration of EP₃ receptor expression in three cell lines. These findings suggest that the DNA sequence of the EP₃ receptor may be methylelated but further studies are needed to clarify whether hypermethylation of the EP₃ receptor gene occurs and regulates EP₃ expression in colon cancers.

In conclusion, data obtained in our present and previous studies suggest that the PGE₂ receptor subtype EP₃ plays an important role in suppression of cell growth and that its downregulation enhances colon carcinogenesis at a later stage. The underlying mechanisms clearly warrant further investigation.

ACKNOWLEDGEMENTS

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EDITOR’S QUIZ: GI SNAPSHOT

Answer

From question on page 1150

An emergency operation was performed which revealed foreign material which had penetrated into the ileum. A wedge resection of the perforated bowel region was undertaken, and intraperitoneal drainage was performed. The patient was discharged from our hospital nine days postoperatively in good condition.

The object that had been imaged on the computed tomography scan was found to be the foot of a soft shelled turtle (fig 2), commonly referred to as “Supon” in Japanese (scientific name *Trionyx sinensis*). This turtle is only served on special occasions and is an expensive item for cuisine. Discussions with the patient indicated that he had eaten soft shelled turtle two months before the operation during a new year festival in January. As an aid in identifying this type of situation, it is important to also make use of preoperative computed tomography scans, review the patient’s history in light of any prior operations and, where possible, evaluate the patient’s menu or discuss with the family to recollect any sources of hard body parts that could be an immediate source of the problem.

Figure 2 A picture of the foot of a soft shelled turtle.

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