COLORECTAL CANCER

Downregulation of prostaglandin E receptor subtype EP_3 during colon cancer development

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Background and aims: Involvement of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, and EP_4 in the formation of aberrant crypt foci (ACF) and/or intestinal polyps has been suggested. In contrast, EP_3 appears to have no influence on the early stages of colon carcinogenesis. In the present study, we examined expression of PGE_2 receptor subtypes EP_1, EP_2, EP_3, and EP_4 in normal colon mucosa and colon cancers, and assessed the contribution of EP_3 to colon cancer development.
Methods: mRNA expression of PGE_2 receptor subtypes EP_1, EP_2, EP_3, and EP_4 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_3 was performed by intraperitoneal injection of AOM, using EP_3 receptor knockout mice. Effects of EP_3 receptor activation on cell growth of human colon cancer cell lines were examined using ONO-AE-248, an EP_3 selective agonist. Moreover, EP_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_1 and EP_2 mRNA were increased in cancer tissues. EP_4 mRNA was constantly expressed in normal mucosa and cancers. In contrast, expression of EP_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_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_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_3 mRNA was detected in only one of 11 human colon cancer cell lines tested. Treatment with 5 μM of an EP_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_3 was expressed. Treatment with 5-aza-dC restored EP_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_3 to some extent.
Conclusion: The PGE_2 receptor subtype EP_3 plays an important role in suppression of cell growth and its downregulation enhances colon carcinogenesis at a later stage. Hypermethylation of the EP_3 receptor gene could occur and may contribute towards downregulating EP_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 humans.[1] Chemically induced colon carcinogenesis in rodents is also suppressed by administration of NSAIDs.[2–4] Moreover, intestinal polyp formation in familial adenomatous polyposis coli patients is markedly reduced after application of agents such as sulindac or indomethacin.[5–8] 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.[9] 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.[1 4 10]

Prostanoids such as prostaglandin (PG)E_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 exert their biological actions through binding to nine specific receptors with seven transmembrane domains: the four subtypes EP_1–EP_4 for PGE_2, DP and CRTH2 for PGD_2, FP for $PGF_{2\alpha}$, IP for PGI_2, and TP for TXA_2.[11 12] Several reports have demonstrated increased levels of PGE_2 in human colon cancer tissues compared with surrounding normal mucosa.[13–15] Signal transduction pathways of PGE_2 receptors have been studied by examining agonist induced changes in the levels of second messengers such as cAMP and free Ca^{2+} and by identifying G protein coupling by various methods.[11] The EP_1 receptor is known to mediate PGE_2 induced elevation of free Ca^{2+} concentration although the species of G protein to which EP_1 receptor is coupled remains unidentified. EP_2 and EP_4 receptors are coupled to Gs and stimulate cAMP production by adenylate cyclase. In contrast, the major signalling pathway for the EP_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.[16]

Abbreviations: PGE_2, prostaglandin E_2; ACF, aberrant crypt foci; AOM, azoxymethane; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; RT-PCR, reverse transcription-polymerase chain reaction; 5-aza-dC, 5-aza-2′-deoxycytidine; FBS, fetal bovine serum

Establishment of mice lacking the genes encoding prostanoid receptors has promoted understanding of the involvement of prostanoids[11] and their receptors in the development of colon cancer.[16–18] In previous studies, we demonstrated that deficiency of either EP_1 or EP_4 receptor decreases formation of azoxymethane (AOM) induced aberrant crypt foci (ACF), putative preneoplastic lesions in the colon.[17 18] Moreover, antagonists of EP_1 and EP_4 receptors suppress formation of AOM induced ACF in the colon of mice and intestinal polyp formation in *Apc* gene deficient Min mice.[17 18] Recently, it was also reported that homozygous deletion of the gene encoding the EP_2 receptor resulted in a decrease in intestinal polyp formation in *Apc* knockout mice.[19] As already mentioned, EP_2 and EP_4 stimulate adenylate cyclase whereas EP_3 exerts an inhibitory influence, suggesting a possible suppressive role against colon carcinogenesis. However, deficiency of EP_3 did not affect AOM induced ACF formation in our previous study.[17]

In the present study, we hypothesised that EP_3 might act at a later stage in colon carcinogenesis. Examination of mRNA expression for EP_1, EP_2, EP_3, and EP_4 in colon carcinomas of mice, rats, and humans demonstrated that levels of EP_3 were markedly decreased compared with normal mucosa. An increase in colon carcinoma formation induced by AOM was also demonstrated in EP_3 receptor knockout mice. Furthermore, activation of the EP_3 receptor showed a suppressive effect on cell growth in a colon cancer cell line in which EP_3 was expressed. In most human colon cancer cell lines tested, EP_3 expression was not detected but treatment with 5-aza-2′-deoxycytidine (5-aza-dC) restored EP_3 expression in some cell lines. On the basis of the results obtained, the role of the EP_3 receptor in colon carcinogenesis is discussed.

MATERIALS AND METHODS

Animals

The mouse gene encoding the PGE_2 receptor EP_3 was disrupted by a gene knockout method using homologous recombination, as reported previously.[17] The generated chimeric mice were backcrossed with C57BL/6Cr mice, and the resulting homozygous mutant mice of these F2 progeny were backcrossed into the C57BL/6Cr background for 10 generations. EP_3 receptor deficient male mice were used at six weeks of age. Genotypes of the knockout mice were confirmed by polymerase chain reaction (PCR) according to the method described previously.[17] Animals were housed in plastic cages at 24±2℃ and 55% relative humidity with a 12 h/12 h light/dark cycle. Water and basal diet (AIN-76A; Bio-Serv, Frenchtown, New Jersey, USA) were given ad libitum. Body weights and food intake were measured weekly.

Colon tumour samples and cell lines

Mouse colon tumours and normal colon mucosa tissues were obtained from C57BL/6J male mice treated with AOM, as previously reported.[18] Rat colon tumours and normal colon mucosa tissues were obtained from eight F344 male rats treated with AOM, as previously reported.[20] 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 adenocarcinoma cell line, was kindly provided by Dr Susan Kirkland, Imperial College of Science, Technology, and Medicine (London, UK).[21] 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℃ in 5% CO_2. Colo 201, DLD-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℃ for 20 seconds, 60℃ for 30 seconds, and 72℃ 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_3* 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 additionally 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 10^2–10^8 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.[20] As the primary antibody, a polyclonal rabbit anti-EP_3 antibody raised against rat EP_3 receptors was used at a 50× dilution.[22] 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.), 3,3′-diaminobenzidine, and hydrogen

Table 1 List of primers used for reverse transcription-polymerase chain reaction

Gene name		Source	Forward primer (5′→3′)		Reverse primer (3′→5′)		Product size (bp)	Cycle No
β-Actin	Mu	NM_007393	AACACCCCAGCCATGTACG	(Exon 4)	CGCTCAGGAGGAGCAATGA	(Exon 6)	623	22
	Rat	NM_031144	AACACCCCAGCCATGTACG	(Exon 4)	CGCTCAGGAGGAGCAATGA	(Exon 6)	623	18
	Hu	NM_001101	AACACCCCAGCCATGTACG	(Exon 4)	CGCTCAGGAGGAGCAATGA	(Exon 6)	623	21
EP_1	Mu	NM_013641	GACGATTCCGAAAGACCGCAG	(Exon 2)	CAACACCACCAACACCAGCAG	(Exon 2 to 3)	242	32
	Rat*	D88751	GAGAACGCAGGTCCCGATG	(Exon 1)	CCAACACCACCAATACCAGCAG	(Exon 1)	232	35
	Hu	NM_000955	GGTATCATGGTGGTGTCGTG	(Exon 2)	GGCCTCTGGTTGTGCTTAGA	(Exon 3)	317	40
EP_2	Mu	NM_008964	GATGGCAGAGGAGACGGAC	(Exon 1)	ACTGGCACTGGACTGGGTAGA	(Exon 2)	295	28
	Rat	NM_031088	TGCTCATCGTGGCTGTGCTC	(Exon 1)	GCTCTCAGTGAAGTCCGACAAC	(Exon 2)	394	35
	Hu	NM_000956	CCACCTCATTCTCCTGGCTA	(Exon 1)	CGACAACAGAGGACTGAACG	(Exon 2)	216	34
EP_3	Mu	D10204	TGCTGGCTCTGGTGGTGAC	(Exon 1)	ACTCCTTCTCCTTTCCCATCTGTG	(Exon 2)	258	30
	Rat	D14869	CCTTTGCCTCCGCCTTCG	(Exon 1)	CGAACGGCGATTAGGAAGG	(Exon 2)	313	35
	Hu	D38297	CTTCGCATAACTGGGGCAAC	(Exon 1)	TCTCCGTGTGTGTCTTGCAG	(Exon 2)	300	35
EP_4	Mu	BC011193	CTGGTGGTGCTCATCTGCTC	(Exon 2)	AGGTGGTGTCTGCTTGGGTC	(Exon 3)	445	30
	Rat	NM_032076	GCCTCAGTGACTTTCGCCG	(Exon 1)	GCTGTGCTGAACCGTCTCTG	(Exon 2)	336	35
	Hu	NM_000958	TGGTATGTGGGCTGGCTG	(Exon 2)	GAGGACGGTGGCGAGAAT	(Exon 3)	329	35

*Rat EP_1 primers were designed to generate no amplicons from either EP_1 variant cDNA (unspliced EP_1 mRNA, Genbank D88752) or genomic DNA.[39]
Mu, mouse; Hu, human.

peroxide. Sections were counterstained with haematoxylin. As a negative control, the primary antibody was preincubated with a 16-fold (molar ratio) excess amount of the fusion protein used as the immunogen for one hour at room temperature prior to incubation of the sections.[22]

AOM induced colon tumour development in EP_3 receptor knockout mice

Male EP_3 receptor deficient homozygous mice ($EP_3^{-/-}$) and wild-type mice received AOM at a dose of 10 mg/kg body weight intraperitoneally once a week for six weeks. At 56 weeks of age, mice were sacrificed under ether euthanasia and complete autopsy was performed. After laparotomy, the entire intestines were resected and opened longitudinally, and the contents were flushed with normal saline. Using a dissection microscope, colon tumours were noted grossly for their location, number, and diameter, measured with callipers. All tumours from AOM treated mice were subjected to histological examination after routine processing and haematoxylin and eosin staining. The experimental protocol was according to the guidelines for Animal Experiments in the National Cancer Center.

Effects of ONO-AE-248 on growth of colon cancer cells

The EP_3 receptor selective agonist 16-(3-methoxymethyl)-phenyl-ω-tetranor-3,7-dithiaPGE$_1$ (ONO-AE-248) was chemically synthesised at Ono Pharmaceutical Co. Ltd.[23] DLD-1 and HCA-7 cells were seeded in plastic 96 well plates at a density of 2×10^3 cells per well, and grown for 24 hours with media containing 5% FBS. The EP_3 receptor selective agonist ONO-AE-248 was added daily on days 0–4, and then numbers of viable cells on day 1, 3, and 5 were measured by colorimetric assay using the cell proliferation assay reagent WST-1 (Wako Chemicals, Osaka, Japan) with a microplate reader (Bio Rad, Hercules, California, USA) at a reference wavelength of 655 nm and a test wavelength of 450 nm. Cell viability was determined as per cent of control values. Experiments were repeated three times and data were measured six times (n = 6).

5′-Aza-2′-deoxycytidine treatment

CACO-2, CW-2, DLD-1, HCA-7, and WiDr cells were seeded at a density of 5×10^4 cells/10 cm dish on day 0 and treated with 1 and 2 μM 5-aza-dC (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 RNA was prepared using Isogen on day 7.

Statistical analysis

The significance of differences in the incidences of tumours was analysed using the χ^2 test and other differences using the Student's *t* test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Different expression of PGE_2 receptors EP_1, EP_2, EP_3, and EP_4 in normal colon mucosa and colon tumours

Expression of PGE_2 receptors EP_1, EP_2, EP_3, and EP_4 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 EP_1 and EP_2 receptor mRNAs was increased compared with levels in normal mucosa. EP_4 mRNA was equally expressed in carcinomas and normal mucosa. In contrast, expression of EP_3 mRNA was markedly decreased in all carcinoma samples compared with normal colon mucosa (fig 1A). Expression patterns of EP_1, EP_2, EP_3,

Table 2 List of primers used for real time reverse transcription-polymerase chain reaction

Gene name		Primer sequences (5′→3′)			Product size (bp)	Cycle condition
β-Actin	Mu, Rat, Hu	Forward	CTACAATGAGCTGCGTGTG	(Exon 3)	122	95°C (20 s) → 60°C (20 s) → 72°C (10 s)
		Reverse	TGGGGTGTTGAAGGTCTC	(Exon 4)		
EP_3	Mouse	Forward	GCTGTCCGTCTGTTGGTC	(Exon 1)	100	95°C (3 s) → 60°C (20 s)
		Reverse	CCTTCTCCTTTCCCATCTG	(Exon 2)		
	Rat	Forward	ACTGTCCGTCTGCTGGTC	(Exon 1)	100	95°C (3 s) → 60°C (20 s)
		Reverse	CCTTCTCCTTTCCCATCTG	(Exon 2)		
	Human	Forward	GTGCTGTCGGTCTGCTG	(Exon 1)	102	95°C (3 s) → 66°C (20 s)
		Reverse	CTTTCTGCTTCTCCGTGTG	(Exon 2)		

Mu, mouse; Hu, human.

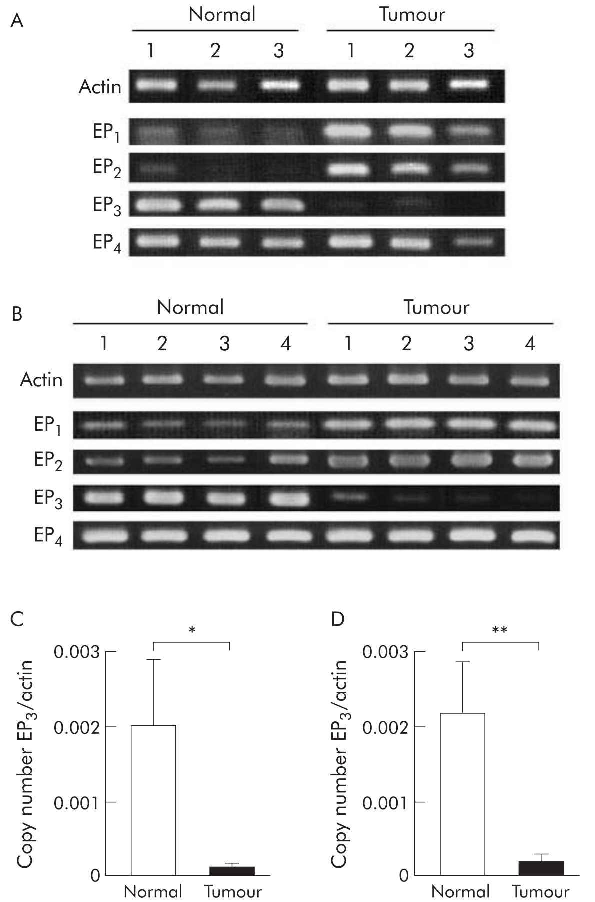

Figure 1 Analyses of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 mRNA expression. (A) Azoxymethane (AOM) treated mouse normal colon mucosa and colon carcinomas. Two pairs of samples (lanes 1, 2) and two independent samples (lane 3) were examine by reverse transcription-polymerase chain reaction (RT-PCR). (B) AOM treated rat normal colon mucosa and colon carcinomas. Four pairs of samples (lanes 1–4) were examined by RT-PCR. Expression levels of EP_3 receptor mRNA were markedly lower in adenocarcinomas than in normal mucosa in all cases. (C, D) Quantitative real time RT-PCR analysis revealed significant downregulation of EP_3 receptor mRNA in AOM treated mice (C) and rat (D) colon carcinomas compared with normal colon mucosa (mouse, n = 3; rat, n = 4). EP_3 receptor mRNA expression was downregulated in tumours, being 5% in the mouse and 9% in the rat of the average value of that in the respective normal colon mucosa. Values are mean (SD); $^*p<0.05$, $^{**}p<0.01$. (A–D) β-Actin was used as an internal control. PCR primers of mouse and rat EP_3 receptors were designed to target a sequence common to all EP_3 receptor variants expressed in each species.

and EP_4 receptors in eight pairs of samples of adenocarcinoma and normal mucosa from AOM treated rats were similar to those in mice. Patterns for EP_1, EP_2, EP_3, and EP_4 receptors in four typical pairs of samples are shown in fig 1B. In the case of human colon tissues, EP_3 receptor mRNA was markedly decreased in seven of eight samples for adenocarcinomas compared with adjacent normal mucosa of the colon. Expression levels of EP_2 receptor mRNA were increased in seven of eight human colon adenocarcinomas compared with levels in normal mucosa, but expression of EP_1 receptor was not clearly increased in human colon carcinoma. EP_4 mRNA was equally expressed in carcinomas and normal mucosa in all cases. Figure 2A shows expression of EP_1, EP_2, EP_3, and EP_4 receptors of colon carcinoma and normal mucosa in four typical pairs of samples.

Furthermore, downregulation of EP_3 was confirmed by quantitative real time RT-PCR (figs 1C, 1D, 2B, 2C).

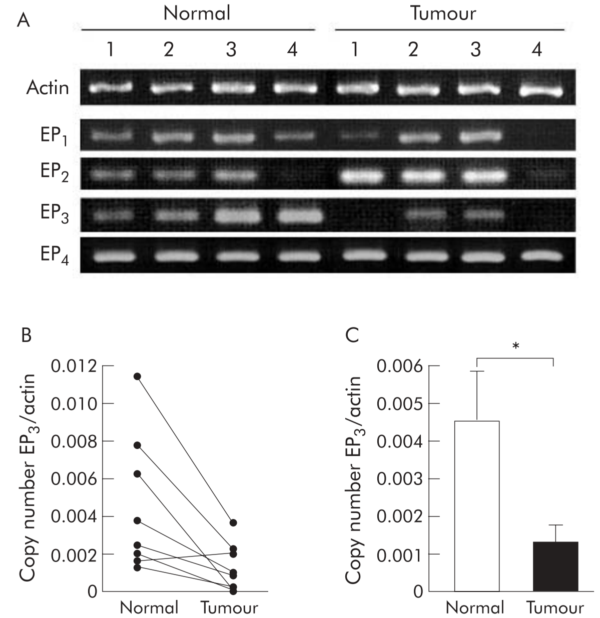

Figure 2 Analyses of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 mRNA expression in human colon tissues. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis patterns in four typical pairs of samples (lanes 1–4) are shown. (B, C) Quantitative real time RT-PCR analysis revealed significant downregulation in EP_3 receptor mRNA. (B) EP_3 receptor mRNA was markedly decreased in seven of eight samples of adenocarcinomas compared with adjacent normal mucosa of the colon. (C) EP_3 receptor mRNA expression was downregulated in tumours, being 28% of the average value of that in adjacent normal colon mucosa. Values are mean (SD); $^*p<0.05$. (A–C) β-Actin was used as an internal control. PCR primers of human EP_3 receptors were designed to target a sequence common to all EP_3 receptor variants expressed.

Expression of EP_3 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 EP_3 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 EP_3 receptor antibody (fig 3A, B) and those stained with anti-EP_3 receptor antibody preabsorbed with fusion EP_3 receptor protein (fig 3C, D). Moreover, slight non-specific staining was detected in red blood cells. In normal colon mucosa tissues, EP_3 receptor expression was prominent in epithelial cells (fig 3E), and the muscular coat was also positively stained. Similarly, positive staining of EP_3 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 EP_3 receptor knockout mice

To assess the role of EP_3 receptors in colon tumour development, EP_3 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

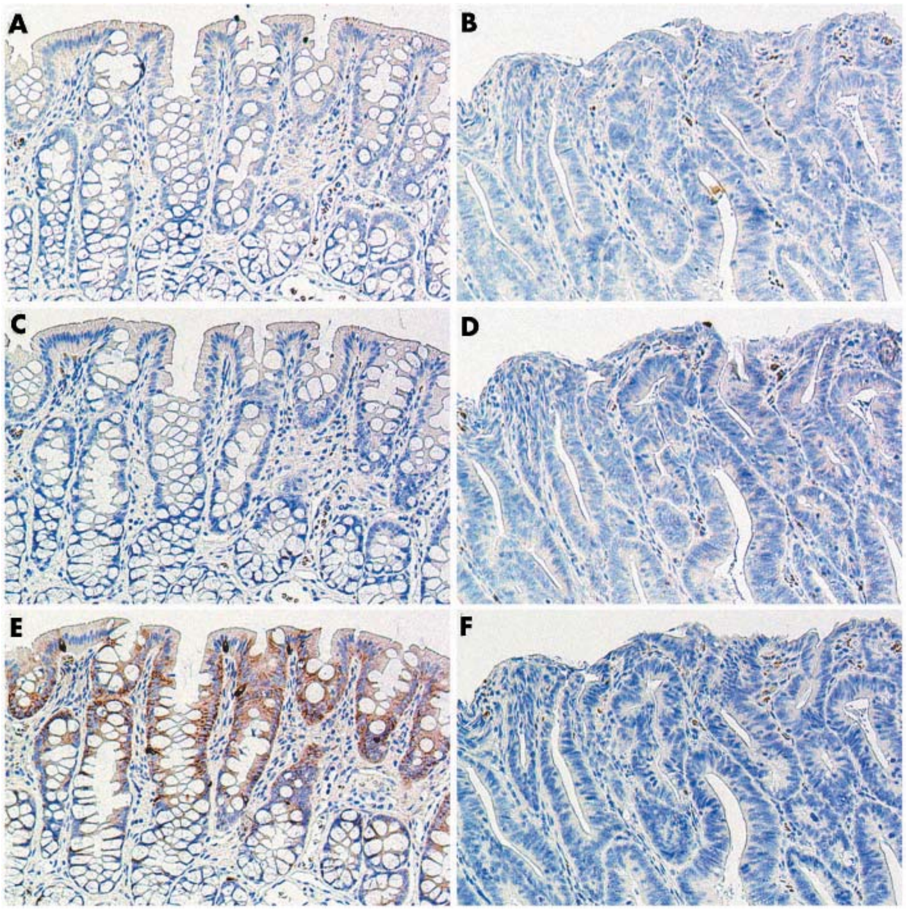

Figure 3 Immunohistochemical staining for the rat prostaglandin E_2 receptor subtype EP_3 of normal colon mucosa (A, C, and E) and colon adenocarcinoma (B, D, and F). Non-specific staining of some red blood cells and weak background staining were observed in the negative controls stained without anti-EP_3 receptor antibody (A, B) and in the negative controls stained with preabsorbed anti-EP_3 receptor antibody (C, D). With anti-EP_3 receptor antibody, immunoreactive EP_3 receptors were prominent in epithelial cells of normal colon mucosa (E) but no EP_3 receptor immunoreactivity was apparent in a colon adenocarcinoma (F). Magnification ×100.

mouse) of colon tumours induced by AOM are summarised in table 3. Tumour incidence was increased to 78% in EP_3 receptor knockout mice compared with 57% in wild-type mice. Regarding tumour multiplicity, values were 2.17 (0.51) for EP_3 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 EP_3 receptor knockout mice. Figure 4 shows the size distribution, demonstrating a significant increase in tumours measuring ⩾2.0 mm in diameter in EP_3 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)).

Table 3 Colon tumour development in EP_3 receptor knockout mice

Mice	Incidence†	Multiplicity‡
Wild-type	16/28 (57%)	0.75 (0.15)
$EP_3^{-/-}$	18/23 (78%)	2.17 (0.51)*

†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 ($p<0.05$).

Expressions of PGE_2 receptors in colon cancer cell lines, and effects of the EP_3 selective agonist on growth of colon cancer cells

Expression of PGE_2 receptors in 11 human colon cell lines was examined by RT-PCR. EP_1, EP_2, and EP_4 were widely detected in the human colon cancer cell lines (in 10 of 11 for EP_1, nine of 11 for EP_2, and nine of 11 for EP_4) but EP_3 was only detected in HCA-7 (fig 5A).

To evaluate the physiological functions of the EP_3 receptor, the effect of an EP_3 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 EP_3 receptor and other PGE_2 receptors (EP_1, EP_2, and EP_4) 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 EP_3 mRNA. The experiments were repeated three times and similar results were obtained.

Effect of 5-aza-dC on EP_3 expression

To determine whether silencing by DNA methylation could be involved in reduced expression of EP_3 receptor in colon tumours, we tested the effects of 5-aza-dC, a demethylating

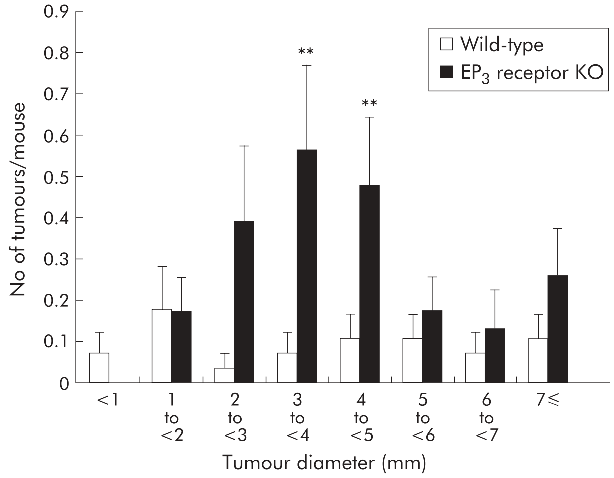

Figure 4 Size distribution of colon tumours induced by azoxymethane in wild-type and prostaglandin E_2 receptor subtype EP_3 knockout (KO) mice. The number of tumours/mouse in each size class is expressed as mean (SEM). **Significantly different from the corresponding wild-type value ($p<0.01$).

agent, on EP_3 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 EP_3 receptor were analysed by RT-PCR. Without 5-aza-dC treatment, expression of EP_3 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 EP_1, EP_2, EP_3, and EP_4 receptors in colon tissues in mice, rats, and humans by RT-PCR and quantitative RT-PCR provided evidence of a marked reduction in EP_3 receptors in colon cancers, in clear contrast with the increase observed for EP_1 and EP_2. 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 tumours with exogenous administration of PGE_2 in male F344 rats, and that colon tumours exhibited similar expression patterns in EP receptors as those observed in the present study.[24] Sonoshita *et al* reported that mRNA expression of EP_2 was strongly increased and EP_3 was weakly decreased in colon polyps compared with normal colon in $APC^{\Delta 716}$ mice.[19] These reports support our data that downregulation of EP_3 is a common feature in colon cancer of mice, rats, and humans. It has been reported that expression of the EP_3 receptor is widely distributed throughout the body, and its mRNA has been identified in almost all tissues in mice and rats, as well as in humans.[25–27] Northern blot analysis revealed that expression of EP_3 receptor mRNA was mainly localised in the muscle layer in the rat gastrointestinal tract,[27] and the present immunohistochemical analysis indicated that EP_3 receptors were detectable in rat normal colon epithelial cells and the muscular coat, but not in rat colon adenocarcinomas. In our previous study, we demonstrated that deficiency of EP_1 or EP_4 receptor reduced formation of AOM induced ACF while EP_3 receptors had no effect, using eight types of EP receptor knockout mice.[17 18] However, long term in vivo examination of AOM induced colon tumour development using EP_3 receptor knockout mice, conducted here in the present study, demonstrated enhancement of tumour incidence and multiplicity.

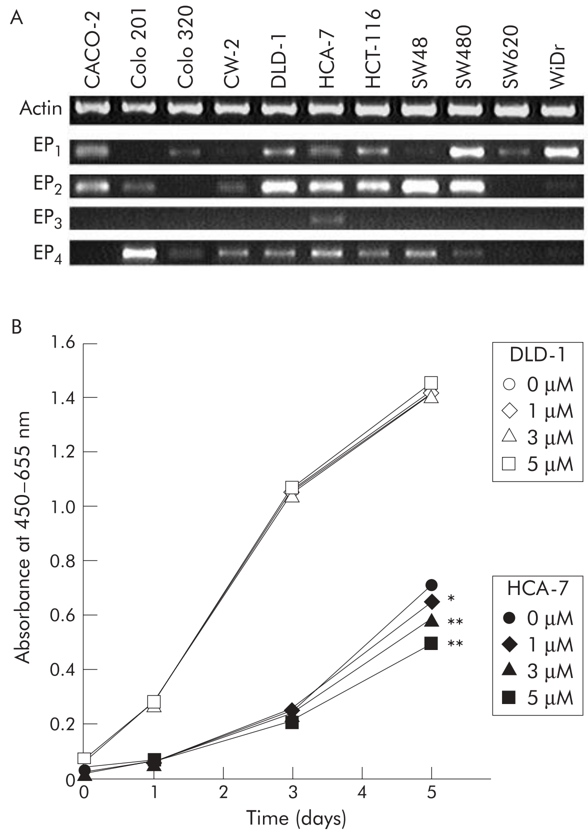

Figure 5 Effect of ONO-AE-248 treatment on cell growth of DLD-1 and HCA-7 cells. (A) Expression of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 was analysed by reverse transcription-polymerase chain reaction in 11 human colon cancer cell lines. (B) DLD-1 and HCA-7 cells were seeded onto 96 well plates at a density of 2×10^3 cells/well, with media containing 5% fetal bovine serum, and treated with the EP_3 receptor selective agonist ONO-AE-248 on days 0-4. Then, cell numbers were measured by WST-1 assay on days 1, 3, and 5. Open symbols indicate DLD-1 and closed symbols HCA-7 cells; concentrations of ONO-AE-248 treatment are indicated (µM). Data are means (n = 6). *$p<0.05$, **$p<0.01$.

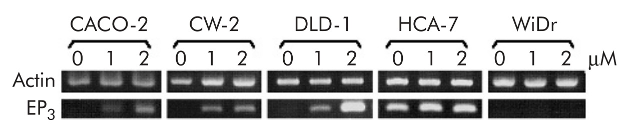

Figure 6 5-Aza-2′-deoxycytidine (5-aza-dC) treatment of CACO-2, CW-2, DLD-1, HCA-7, and WiDr colon cancer cell lines. Each cell line was treated with 1 and 2 µM 5-aza-dC three times. EP_3 receptor expression was analysed by reverse transcription-polymerase chain reaction.

Moreover, the size of the tumours was significantly increased. Thus based on our present and previous results, we suggest that the EP_3 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 EP_3 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 EP_3 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.[30 31] Two of the three variants of the mouse EP_3 receptors are $EP_{3\alpha}$ and $EP_{3\beta}$, which are coupled to G_i and cause inhibition of adenylate cyclase.[30] The mouse $EP_{3\gamma}$ receptor, in contrast, is coupled to G_s, in addition to G_i, and evokes pertussis toxin insensitive cAMP production.[31] Preliminarily, we examined expression of three splice variants of mouse EP_3 receptors by RT-PCR using specific primers for each variant, and found $EP_{3\alpha}$ to be the major form in mouse normal mucosa (data not shown). These observations support the conclusion that the major splice variants of EP_3 receptors are coupled to G_i and act to inhibit adenylate cyclase in normal colon mucosa in mice. On the other hand, EP_2 and EP_4 receptors are coupled to G_s 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.[34] In our previous study,[18] the EP_4 receptor selective agonist ONO-AE1-329 was shown to enhance colony formation by the HCA-7 human colon adenocarcinoma cell line. The EP_3 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 $EP_{3\alpha}$ receptor.[23] Therefore, the EP_3 receptor pathway may play an important role in counteracting the effects of EP_2 and EP_4 receptors, and its downregulation in later stages of colon carcinogenesis may enhance cancer development. Additional studies are needed to investigate interactions between the EP_3 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,[35 36] and silencing of *COX-2* and *APC* genes by hypermethylation has been reported in human colon cancer.[37 38] Although hypermethylation of the prostaglandin receptor gene has not been reported,[37 38] DNA sequences in the promoter region and exon 1 of the human EP_3 gene are GC rich (Genbank AL031429). Therefore, in the present study, we examined the effects of demethylation of DNA with 5-aza-dC on EP_3 expression in human colon cancer cell lines. Demethylation of five cell lines by 5-aza-dC treatment resulted in restoration of EP_3 receptor expression in three cell lines. These findings suggest that the DNA sequence of the EP_3 receptor may be methylated but further studies are needed to clarify whether hypermethylation of the EP_3 receptor gene occurs and regulates EP_3 expression in colon cancers.

In conclusion, data obtained in our present and previous studies suggest that the PGE_2 receptor subtype EP_3 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.

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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 post-operatively 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.

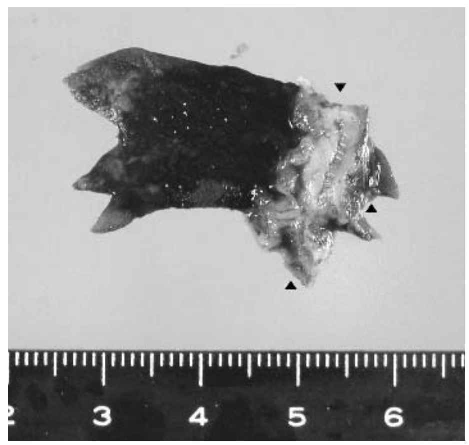

Figure 2 A picture of the foot of a soft shelled turtle.

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