32. Peterson, J.R., and Mitchison, T.J. (2002). Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton. Chem. Biol. *9*, 1275–1285.
33. Crews, C.M., and Splittgerber, U. (1999). Chemical genetics: exploring and controlling cellular processes with chemical probes. Trends Biochem. Sci. *24*, 317–320.
34. Root, D.E., Flaherty, S.P., Kelley, B.P., and Stockwell, B.R. (2003). Biological mechanism profiling using an annotated compound library. Chem. Biol. *10*, 881–892.
35. Schreiber, S.L. (2000). Target-oriented and diversity-oriented organic synthesis in drug discovery. Science *287*, 1964–1969.
36. Kuruvilla, F.G., Shamji, A.F., Sternson, S.M., Hergenrother, P.J., and Schreiber, S.L. (2002). Dissecting glucose signalling with diversity-oriented synthesis and small-molecule microarrays. Nature *416*, 653–657.
37. Ohmori, E., Fukui, T., Imanishi, N., Yatsunami, K., and Ichikawa, A. (1990). Purification and characterization of l-histidine decarboxylase from mouse mastocytoma P-815 cells. J. Biochem. (Tokyo) *107*, 834–839.
38. Shore, P.A., Burkhalter, A., and Cohn, V.H. (1959). A method for the fluorometric assay of histamine in tissues. J. Pharmacol. Exp. Ther. *127*, 182–186.

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Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation

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Abstract

Prostaglandins (PGs) have been shown to play various roles in adipogenesis. In this study, we investigated on which PGE receptor subtypes are involved in the inhibition of 3T3-L1 preadipocyte differentiation. The triglyceride content of cells, used as an index of differentiation, was decreased when PGE_2, the FP-agonist fluprostenol or dibutyryl cAMP, was exogenously added to differentiation cocktails. 3T3-L1 preadipocyte cells express mRNAs for the prostanoid EP4, FP, and IP receptors. PGE_2 and the EP4 agonist AE1–329 increased cAMP levels in preadipocytes in a dose-dependent manner. AE1–329 suppressed the expression induction of differentiation marker genes such as resistin and peroxisome proliferator-activated receptor-γ. The inhibitory effect of PGE_2 but not that of fluprostenol was reversed by the addition of the EP4 antagonist AE3–208. AE3–208 mimicked the differentiation-promoting effects of indomethacin. These results suggest that the EP4 receptor mediates the suppressive action of PGE_2 in 3T3-L1 adipocyte differentiation.

Keywords: Prostanoid; Receptor subtypes; Adipogenesis; Fat cell; Aspirin-like drugs

Adipogenesis is a crucial aspect in controlling body fat mass [1,2]. Acquisition of the mature adipocyte phenotype is a highly regulated process in which preadipocytes undergo differentiation, resulting in both an increase in size and number of mature adipocytes in adipose tissue. It has been shown that cyclooxygenase (COX) products such as prostaglandin (PG) E_2 and $PGF_{2\alpha}$ inhibit adipocyte development [3,4]. A recent study suggested that COX-2 might be involved in body fat regulation [5]. Mice heterozygous for the COX-2 gene showed approximately 30% increased body weight, with 2 to 3-fold larger fat pads compared with those of wild-type animals. PGE_2 production in adipose tissue from COX-2 null mice was only 20% of that of wild-type mice. These results suggested that COX-2 as well as PGE_2 participates in the negative regulation of adipocyte differentiation.

PGs exert a wide range of actions through their binding to plasma membrane receptors [6,7]. $PGF_{2\alpha}$ exerts its actions via a specific interaction with the type F prostanoid receptor FP which activates phospholipase C, resulting in phosphatidylinositol breakdown [8]. In contrast, PGE_2 exerts its actions through its interaction with four PGE_2 receptor subtypes (EP; EP1, EP2, EP3, and EP4). The EP subtypes differ in their signal transduction pathways; EP1 is coupled to the mobilization of intracellular $[Ca^{2+}]$, EP2, and EP4 are coupled to the stimulation of adenylyl cyclase, and EP3 is mainly coupled to inhibition of adenylyl cyclase. The diverse actions of PGE_2 can be explained by the existence of these multiple EP subtypes with different signal transduction pathways [9,10]. Due to the lack of subtype-specific agonists and antagonists, the involvement of each EP

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subtype in a specific PGE_2 action including suppression of adipocyte differentiation has not been well established. The current study was undertaken to determine which EP subtype participates in the negative regulation of adipocyte differentiation. 3T3-L1 cells were used as a model system and a pharmacological approach using a highly selective EP agonist and antagonist was employed to determine the relative contributions of the EP receptor subtypes.

Materials and methods

Reagents. Dibutyryl cAMP, dexamethasone, and indomethacin were purchased from Sigma (St. Louis, MO). The ^{125}I-labeled cyclic AMP assay system was purchased from Amersham Biotech (Piscataway, NJ). PGE_2 was purchased from Funakoshi (Tokyo, Japan), and fluprostenol was from Cayman Chemical (Ann Arbor, MI). DI-004 (an EP1 agonist), AE1-259 (an EP2 agonist), AE1-329 (an EP4 agonist), and AE3-208 (an EP4 antagonist) were generous gifts from ONO Pharmaceuticals (Osaka, Japan) [11–13]. Oligonucleotides were from Invitrogen (Carlsbad, CA). All other chemicals were commercial products of reagent grade.

Cell culture, differentiation, and measurement of triglyceride content. 3T3-L1 preadipocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine. Differentiation was initiated by culturing the cells in differentiation medium which contained 10% FBS, 4 mM glutamine, 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone, and 5 μg/ml insulin. After two days, the culture medium was changed to adipocyte growth medium containing 10% FBS, 4 mM glutamine, and 5 μg/ml insulin and exchanged every two days for an additional six days. Reagents such as PG agonists and the PG antagonist were added to both the differentiation medium and adipocyte growth medium as required. For measurements of triglyceride content, cells grown in six-well plates were harvested in 1 ml of 2-propanol, sonicated, and triglyceride levels in the cell lysate were measured using Triglyceride G Test Kit (Wako, Tokyo, Japan). For Oil Red O staining, cells were washed in PBS, fixed in 3.7% formaldehyde for 10 min, and followed by staining with Oil Red O for 1 h. Oil Red O was prepared by diluting a stock solution (0.5 g of Oil Red O (Sigma) in 100 ml of 2-propanol) with water (6:4) followed by filtration.

cAMP formation assay. cAMP levels in 3T3-L1 cells on day 0 and day 2 of the differentiation program were determined as reported previously [14]. Cells cultured in 24-well plates (1×10^6 cells/well) were washed twice with 0.5 ml Krebs–Hepes buffer (pH 7.4) with 10 μM indomethacin, and preincubated in this solution for 10 min. Reactions were started by the addition of test reagents along with 100 μM Ro-10-1724 and 10 μM indomethacin. After incubation for 10 min at 37 °C, reactions were terminated by the addition of 10% trichloroacetic acid. The cAMP content of the cells was then measured using a cAMP radioimmunoassay kit.

[^{3}H]PGE_2 binding assay. 3T3-L1 preadipocytes grown to confluency were homogenized with a Potter–Elvehjem homogenizer in 10 mM Mes/NaOH, pH 6.0, containing 10 mM $MgCl_2$, 1 mM EDTA, 20 μM indomethacin, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 250,000*g* for 10 min, the pellet was washed and suspended in the same buffer. The membrane fraction (200 μg) was incubated with 4 nM [^{3}H]PGE_2 at 30 °C for 1 h, and [^{3}H]PGE_2 bound to the membrane fraction was determined by adding a 1000-fold excess of unlabeled PGE_2 to the incubation mixture. Specific binding was calculated by subtracting the non-specific binding from the total binding.

RNA isolation and semi-quantitative RT-PCR. Total cellular RNA was isolated from 2×10^6 3T3-L1 cells on the indicated day of the differentiation program by the acid guanidinium thiocyanate–phenol–chloroform method [15]. Cells collected just before being cultured in fresh differentiation cocktail were collected as the sample at time 0 h. To examine the differentiation-dependent gene expression of peroxisome proliferator-activated receptor-γ (PPARγ) and resistin, semi-quantitative reverse transcription PCR (RT-PCR) was performed. Complementary DNA was synthesized from total RNA (10 μg) using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using a GeneAmp 9700 (Perkin–Elmer Applied Biosystems, Foster City, CA). Primers and PCR conditions used in the PCR for EP1, EP2, EP3, EP4, IP, and FP genes have been described previously [16]. Primers used for PPARγ, resistin, and β-actin were as follows: PPARγ, 5′-ttcccagcattttctgctccacactatga ag-3′ (sense), 5′-cggcagttaagatcacacctatcataaaata-3′ (antisense); resistin, 5′-aatgcaataaagaacattggc-3′ (sense), 5′-aggtgcctgtagagaccggag-3′ (antisense); and β-actin, 5′-accaactgggacgacatggagaagatctgg-3′ (sense), 5′-ccggccagccaggtccagacgcaggatggc-3′ (antisense). All PCRs were confirmed to be in the logarithmic phase by monitoring the products obtained at the indicated number ±2 cycles. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The RT-PCR experiments were independently repeated three times.

Results

Effects of fluprostenol, PGE_2, and dibutyryl cAMP on adipocyte differentiation

3T3-L1 preadipocyte cells were primed with insulin, dexamethasone, and IBMX for two days followed by treatment with insulin for an additional six days. Their differentiation into adipocytes was monitored by Oil Red O staining, and furthermore their triglyceride (TG) content was measured as an index of differentiation (Figs. 1A and B). Indeed, the adipocytes contained 80.4 ± 2.94 mg TG/plate (2.0×10^6 cells/plate), but the 3T3-L1 cells cultured in the absence of the differentiation cocktail exhibited only 3.45 ± 0.15 mg TG/plate (data not shown). As previously reported, when the differentiation program was performed in the presence of 0.1 μM of fluprostenol, an FP-agonist, the positive area stained with Oil Red O was greatly reduced and the TG content was reduced to approximately one-fifth of the control level (Figs. 1A and B). Similarly, PGE_2 (0.1 μM) as well as the membrane-permeable cAMP, dibutyryl cAMP, markedly reduced the Oil Red O-stained regions and TG content in 3T3-L1 cells (Figs. 1A and B).

Expression of prostanoid EP4 receptors during adipocyte differentiation

We next examined the mRNA expression of prostanoid receptors in 3T3-L1 cells during the differentiation program (Fig. 2). As previously reported [17], the FP and IP mRNAs were expressed during the differentiation period. In addition, among the PGE receptor subtypes, significant expression of EP4 mRNA was de-

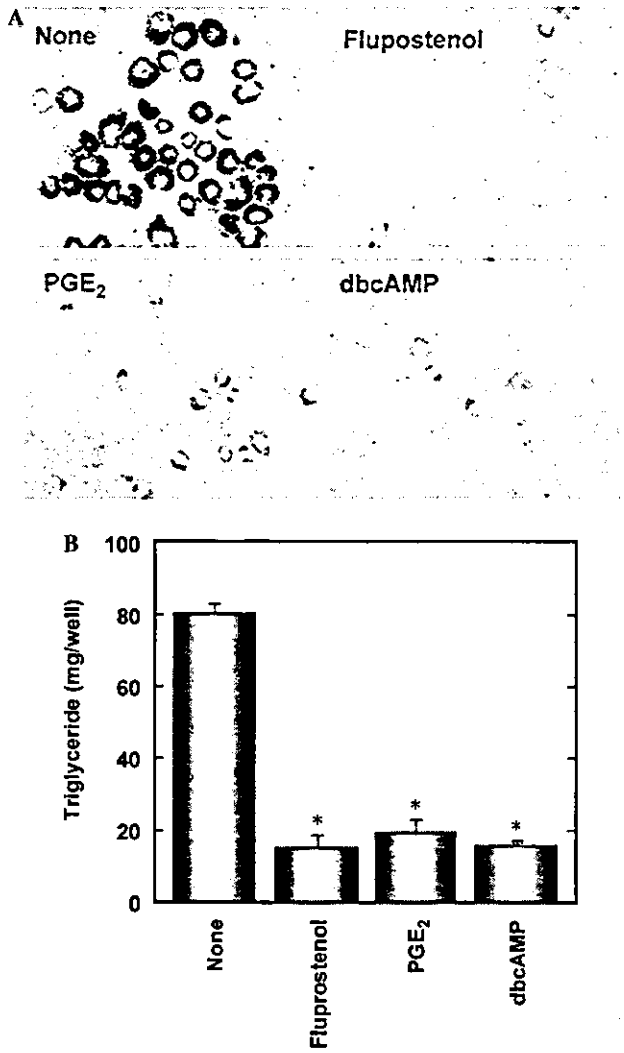

Fig. 1. PGE_2 and dibutyryl cAMP suppress 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with the FP-agonist fluprostenol (0.1 μM), PGE_2 (0.1 μM) or dibutyryl cAMP (dbcAMP) (1 mM). On day 8, the cells were subjected to Oil Red O staining (A), or suspended in 2-propanol, and their triglyceride content was determined as described in Materials and methods (B). Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus vehicle (Student's t test).

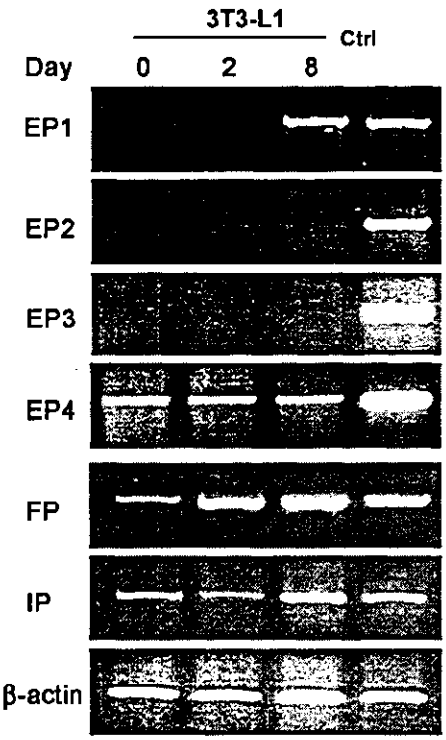

Fig. 2. RNA expression of prostanoid receptors during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were treated with a standard differentiation cocktail as described in Materials and methods. Total RNA was extracted from untreated cells (day 0), cells on day 2 (day 2) or cells on day 8 (day 8) of the differentiation program. Total RNA was subjected to RT-PCR analysis. Mouse lung RNA was used as a positive control for EP2, EP4, IP, and β-actin, and mouse kidney RNA was used for EP1, EP3, and FP (Ctrl). The experiments were independently repeated three times and similar results were obtained.

tected throughout the differentiation process. We failed to detect a significant amount of EP2 and EP3 receptor mRNA in these cells. Expression of EP1 mRNA was undetectable in the untreated cells and in the cells on day 2, but could be detected in cells on day 8. When we performed the $[^3H]PGE_2$ binding assay with a crude membrane fraction of undifferentiated 3T3-L1 preadipocytes, we detected a specific binding with a value of 15.1 ± 1.6 fmol/mg protein, and this binding was inhibited by more than 70% in the presence of an excess of the EP4-specific agonist AE1-329, but not in the presence of an excess of the EP2-specific agonist AE1-259. Based on these results, EP4 appears to be the EP receptor predominantly expressed in 3T3-L1 preadipocytes. We then performed the cAMP formation assay in 3T3-L1 preadipocytes (Fig. 3). PGE_2, but not fluprostenol, significantly increased cAMP formation in these cells in a dose dependent manner. This effect of PGE_2 was mimicked by AE1-329, but not by AE1-259. These results indicate that 3T3-L1 preadipocytes express functional EP4 receptors coupled to the stimulation of cAMP production.

Effects of an EP4 agonist on gene expression of resistin and PPARγ during adipocyte differentiation

When we investigated the effects of EP-selective agonists on adipocyte differentiation, AE1-329 mimicked the inhibitory effect of PGE_2, but DI-004, an EP1 agonist, failed. These results indicated that PGE_2 inhibits adipocyte differentiation by acting on EP4 receptor (Fig. 4A). We then examined the effects of an EP4 agonist on differentiation-dependent gene expression of resistin and PPARγ in 3T3-L1 cells (Fig. 4B). The mRNA for resi-

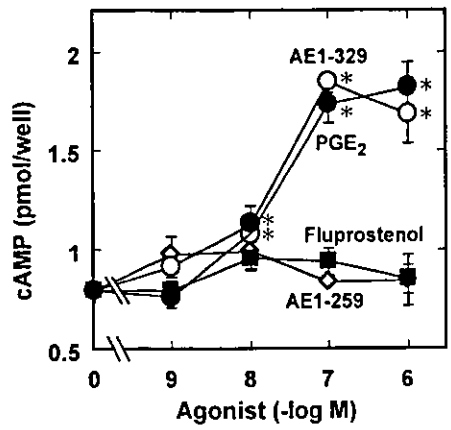

Fig. 3. Effects of PG agonists on cAMP accumulation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes (1×10^6 cells/well) were stimulated with the indicated concentrations of PGE_2, an EP4 agonist (AE1-329), an EP2 agonist (AE1-259), and fluprostenol for 10 min at 37 °C. cAMP formed was measured by radioimmunoassay. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus the basal cAMP level (Student's t test).

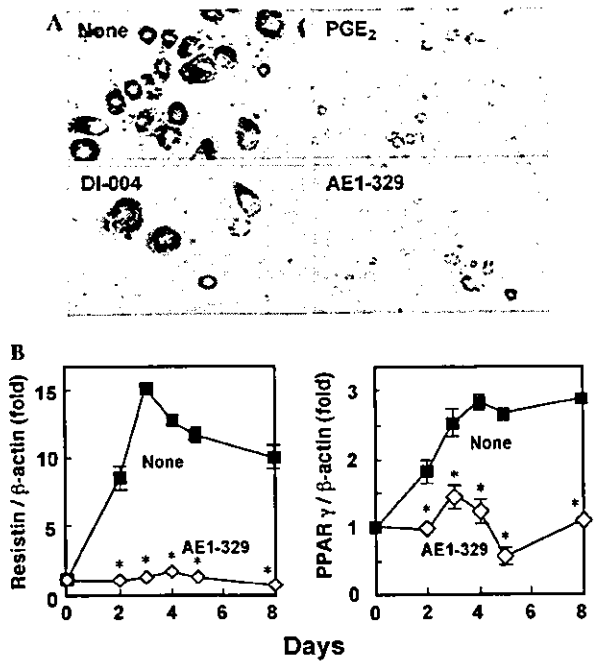

Fig. 4. Effects of an EP4 agonist on adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with PGE_2, DI-004 or AE1-329 (0.1 μM for each reagent). On day 8, the cells were subjected to Oil Red O staining (A). Alternatively, 3T3-L1 cells were stimulated with a standard differentiation cocktail in the presence or absence of AE1-329 (0.1 μM). Total RNA was isolated on the indicated day of the differentiation program, and subjected to semi-quantitative RT-PCR analysis as described in Materials and methods (B). The resistin and PPARγ mRNA levels were normalized to the β-actin mRNA levels and the data are represented as the fold of the value on day 0. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

stin, a hormone linking obesity to diabetes in rodents [18], was only weakly expressed in preadipocytes, but was drastically induced during the first three days of differentiation and its expression remained greater than 10-fold of the basal level until day 8. AE1–329 (0.1 μM) completely inhibited this increase in expression levels, resulting in an almost unchanged level of expression throughout the differentiation period. The mRNA for PPARγ, a transcription factor playing a central role in differentiation [19–21], was induced by 2-fold on the second day, and was subsequently maintained at high levels of greater than 2.5-fold of the basal expression. AE1–329 completely inhibited the increase observed on day 2, and suppressed the expression of PPARγ mRNA to within 1.5-fold of the basal level throughout the differentiation period. Such inhibition of resistin and PPARγ gene expression by AE1–329 was reproduced by PGE_2, but not by AE1–259 (data not shown). These results suggest that EP4 is the receptor responsible for the suppressive effects of PGE_2 on adipogenesis.

PGE_2-elicted but not FP-agonist-elicited inhibition of differentiation is mediated via EP4 receptor activation

It has been reported that FP receptor coupling to intracellular Ca^{2+} mobilization is also involved in the inhibition of adipocyte differentiation [22,23]. To examine the possible involvement of EP4 receptor signaling in the inhibition of adipocyte differentiation and to discriminate it from FP-mediated inhibition, we investigated the effects of an EP4 antagonist on PGE_2- or fluprostenol-elicited inhibition of differentiation (Fig. 5). PGE_2 dose-dependently decreased cellular TG accumulation with an EC_{50} value of 10^{-8} M in the absence of an EP4 antagonist. The EP4 antagonist AE3–208 decreased this inhibition and shifted the dose-dependent curve rightward by two orders. In contrast, fluprostenol also showed an inhibitory effect on differentiation in our system, but this inhibition was insensitive to AE3–208 treatment. These results suggest that PGE_2 exhibits an inhibitory effect on differentiation via EP4 receptor activation, which is unrelated to FP receptor stimulation.

An EP4 antagonist promotes adipocyte differentiation

Endogenous prostanoids have been suggested to be involved in the inhibition of adipocyte differentiation, since COX inhibitors such as indomethacin display enhancing effects on differentiation [24,25]. We therefore investigated whether the differentiation-promoting effects of indomethacin (10 μM) are mimicked by an EP4 antagonist (AE3–208, 1 μM). As reported previously, indomethacin significantly promoted the differentiation of adipocytes as revealed by the increased TG content (Fig. 6). AE3–208 also showed an enhancing

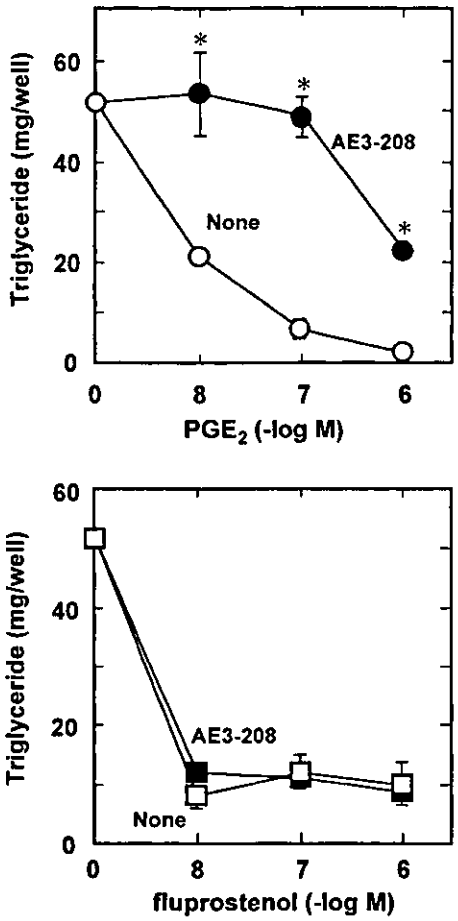

Fig. 5. Effects of an EP4 antagonist on PGE_2- or fluprostenol-elicited inhibition of 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail supplemented with the indicated concentrations of PGE_2 or fluprostenol in the presence (AE3-208) or absence of 1 μM AE3-208 (none). On day 8 of the differentiation program, cells were suspended in 2-propanol, and their triglyceride contents were determined as described in Materials and methods. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

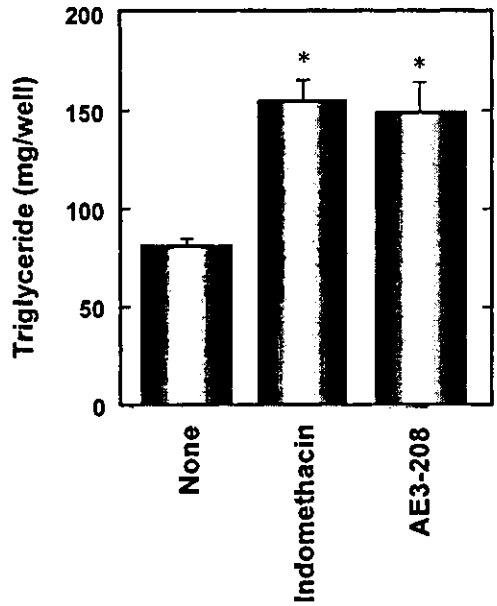

Fig. 6. Effects of indomethacin and an EP4 antagonist on 3T3-L1 preadipocyte differentiation. 3T3-L1 cells grown to confluency (2×10^6 cells/plate) were stimulated with a standard differentiation cocktail (none) or differentiation cocktail supplemented with indomethacin (10 μM) or AE3-208 (1 μM). On day 8 of the differentiation program, cells were suspended in 2-propanol, and their triglyceride contents were determined as described in Materials and methods. Values represent means ± SEM of three independent experiments. $^*P < 0.05$ versus none (Student's t test).

effect on differentiation. These results suggest that endogenously synthesized PGE_2 has a suppressive role in 3T3-L1 differentiation by acting on the EP4 receptor.

Discussion

PGs have long been thought to contribute to fat cell development, but the role of PGs in the regulation of adipocyte differentiation is complex and has remained unclear [4]. One of the reasons for its complexity is that different classes of PGs exert opposing effects on differentiation. For instance, both PGI_2 and PGE_2, the two PGs predominantly synthesized by fat cells, appear to have opposing effects on early adipogenesis; PGI_2 promotes adipocyte differentiation [26,27], whereas PGE_2 inhibits differentiation. PGI_2 exerts its action by acting on the prostanoid IP receptor, while PGE_2 exhibits its versatile actions through its binding to four kinds of EP receptor subtypes with quite different signaling pathways. We hypothesized that the complex role of PGs in adipogenesis may be explained by the expression of multiple EP receptor subtypes in preadipocytes. Indeed, Borglum et al. [17] previously reported the existence of mRNAs for several kinds of PG receptors and EP receptor subtypes in Ob1771 preadipocytes. However, which PGE receptor(s) are functionally involved in the regulation of adipocyte differentiation remained poorly understood. In this study, we focused on identifying the receptor subtype(s) involved in the inhibition of adipocyte differentiation by PGE_2, and demonstrated that EP4 is the receptor responsible for this inhibition. The EP4 receptor is coupled to stimulation of adenylyl cyclase. Indeed, we demonstrated that an EP4 agonist as well as PGE_2 elicits an increase in intracellular cAMP levels in 3T3-L1 preadipocytes. We also showed that the cAMP analogue dibutyryl cAMP inhibits adipocyte differentiation as reported previously [24,28]. These results suggest that EP4 inhibits adipocyte differentiation in a cAMP-dependent manner.

It has been reported that exogenously added arachidonic acid inhibits 3T3-L1 differentiation [29]. When cells were treated with COX inhibitors, the inhibition of

differentiation was reversed, indicating that a COX-derived PG is necessary for arachidonic acid to inhibit differentiation. Both PGE_2 and $PGF_{2\alpha}$ are able to substitute for arachidonic acid when added to the differentiation cocktail [24]. Casimir et al. [22,23] demonstrated that $PGF_{2\alpha}$ as well as an FP-agonist inhibits differentiation more effectively than PGE_2, and thus they proposed that $PGF_{2\alpha}$ is the main PG involved in the inhibition of differentiation and that PGE_2 may only exert its inhibitory action by cross-reacting to the FP receptor. However, the current study demonstrated that the effect of PGE_2 but not that of $PGF_{2\alpha}$ is reversed by an EP4 antagonist. These results suggest that inhibition of adipocyte differentiation by PGE_2 is mediated by the EP4 receptor and is distinct from $PGF_{2\alpha}$ inhibition. Because arachidonic acid is converted more efficiently into PGE_2 than $PGF_{2\alpha}$ in 3T3-L1 cells [30], and because suppression by arachidonic acid is reversed by an inhibitor for protein kinase A [28], the inhibitory effect of arachidonic acid may be mediated through the PGE_2–EP4 pathway.

One of the interesting findings in this study is differentiation-dependent induction of EP1 gene expression. It was reported that the EP1 mRNA is expressed in mature adipocytes isolated from mouse adipose tissue [17]. Since EP1 has been shown to couple with phospholipase C and intracellular Ca^{2+} mobilization, EP1 might also inhibit differentiation as FP receptor does. However, an EP1-agonist failed to elicit significant inhibitory effects on adipocyte differentiation. Differentiation-dependent gene expression suggested that EP1 may be involved in some function of mature adipocytes. Indeed, it was reported that PGE_2 increases the calcium concentration and oxygen consumption in rat brown adipose tissue [31]. PGE_2 may stimulate metabolism of brown adipose tissue via EP1 receptor.

The current study demonstrated that the differentiation-enhancing effects of indomethacin can be mimicked by an EP4 antagonist. This result indicates that EP4 negatively regulates the standard differentiation process of 3T3-L1 cells. Yan et al. [32] reported that both a COX-1- and COX-2-inhibitor enhances differentiation of 3T3-L1 cells, indicating that both COX isozymes participate in the negative regulation of adipogenesis. Interestingly, Yan et al. also demonstrated that COX-2 inhibitors, but not a COX-1 inhibitor, reversed TNF-α-induced inhibition of differentiation. A similar modulating effect of COX-2 has been shown in adiponectin signaling [33]. Involvement of the EP4 signaling pathway in these systems is an interesting issue to examine in the future.

Acknowledgments

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References

[1] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, Physiol. Rev. 78 (1998) 783–809.
[2] E.D. Rosen, B.M. Spiegelman, Molecular regulation of adipogenesis, Annu. Rev. Cell Dev. Biol. 16 (2001) 145–171.
[3] P.B. Curtis-Prior, Prostaglandins and obesity, Lancet 1 (1975) 897–899.
[4] S. Kim, N. Moustaid-Moussa, Secretory, endocrine and autocrine/paracrine function of the adipocyte, J. Nutr. 130 (2000) 3110S–3115S.
[5] J.N. Fain, L.R. Ballou, S.W. Bahouth, Obesity is induced in mice heterozygous for cyclooxygenase-2, Prostaglandins Other Lipid Mediat. 65 (2001) 199–209.
[6] R.A. Coleman, W.L. Smith, S. Narumiya, International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes, Pharmacol. Rev. 46 (1994) 205–229.
[7] S. Narumiya, Y. Sugimoto, F. Ushikubi, Prostanoid receptors; structures, properties and functions, Physiol. Rev. 79 (1999) 1193–1226.
[8] Y. Sugimoto, K. Hasumoto, T. Namba, A. Irie, M. Katsuyama, M. Negishi, A. Kakizuka, S. Narumiya, A. Ichikawa, Cloning and expression of a cDNA for mouse PGF receptor, J. Biol. Chem. 269 (1994) 1356–1360.
[9] M. Negishi, Y. Sugimoto, A. Ichikawa, Prostaglandin E receptors, J. Lipid Mediat. Cell Signalling 12 (1995) 379–391.
[10] Y. Sugimoto, S. Narumiya, A. Ichikawa, Distribution and function of prostanoid receptors: studies from knockout mice, Prog. Lipid Res. 39 (2000) 289–314.
[11] T. Suzawa, C. Miyaura, M. Inada, T. Maruyama, Y. Sugimoto, F. Ushikubi, A. Ichikawa, S. Narumiya, T. Suda, The role of prostaglandin E receptor subtypes (EP1, EP2, EP3 and EP4) in bone resorption: an analysis using specific agonists for the respective EPs, Endocrinology 141 (2000) 1554–1559.
[12] K. Yoshida, H. Oida, T. Kobayashi, T. Maruyama, M. Tanaka, T. Katayama, K. Yamaguchi, E. Segi, T. Tsuboyama, M. Matsushita, K. Ito, Y. Ito, Y. Sugimoto, F. Ushikubi, S. Ohuchida, K. Kondo, T. Nakamura, S. Narumiya, Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation, Proc. Natl. Acad. Sci. USA 99 (2002) 4580–4585.
[13] K. Kabashima, T. Saji, T. Murata, M. Nagamachi, T. Matsuoka, E. Segi, K. Tsuboi, Y. Sugimoto, T. Kobayashi, Y. Miyachi, A. Ichikawa, S. Narumiya, The prostaglandin E receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut, J. Clin. Invest. 109 (2002) 883–893.
[14] N. Hatae, K. Yamaoka, Y. Sugimoto, M. Negishi, A. Ichikawa, Augmentation of receptor-mediated adenylyl cyclase activity by Gi-coupled prostaglandin receptor subtype EP3 in a Gbetagamma subunit-independent manner, Biochem. Biophys. Res. Commun. 290 (2002) 162–168.
[15] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[16] E. Segi, K. Haraguchi, Y. Sugimoto, M. Tsuji, H. Tsunekawa, S. Tamba, K. Tsuboi, S. Tanaka, A. Ichikawa, Expression of messenger RNA for prostaglandin E receptor subtypes EP4/EP2

and cyclooxygenase isozymes in mouse periovulatory follicles and oviducts during superovulation, Biol. Reprod. 68 (2003) 804–811.
[17] J.D. Borglum, S.B. Pedersen, G. Ailhaud, R. Negrel, B. Richelsen, Differential expression of prostaglandin receptor mRNAs during adipose cell differentiation, Prostaglandins Other Lipid Mediat. 57 (1999) 305–317.
[18] C.M. Steppan, S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, M.A. Lazar, The hormone resistin links obesity to diabetes, Nature 409 (2001) 292–293.
[19] O.A. MacDougald, M.D. Lane, Transcriptional regulation of gene expression during adipocyte differentiation, Annu. Rev. Biochem. 64 (1995) 345–373.
[20] P. Tontonoz, E. Hu, B.M. Spiegelman, Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma, Curr. Opin. Genet. Dev. 5 (1995) 571–576.
[21] P.D. Miles, Y. Barak, W. He, R.M. Evans, J.M. Olefsky, Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency, J. Clin. Invest. 105 (2000) 287–292.
[22] C.W. Miller, D.A. Casimir, J.M. Ntambi, The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2alpha, Endocrinology 137 (1996) 5641–5650.
[23] D.A. Casimir, C.W. Miller, J.M. Ntambi, Preadipocyte differentiation blocked by prostaglandin stimulation of prostanoid FP2 receptor in murine 3T3-L1 cells, Differentiation 60 (1996) 203–210.
[24] I.H. Williams, S.E. Polakis, Differentiation of 3T3-L1 fibroblasts to adipocytes. The effect of indomethacin, prostaglandin E1 and cyclic AMP on the process of differentiation, Biochem. Biophys. Res. Commun. 77 (1977) 175–186.
[25] P. Verrando, R. Negrel, P. Grimaldi, M. Murphy, G. Ailhaud, Differentiation of ob 17 preadipocytes to adipocytes. Triggering effects of clofenapate and indomethacin, Biochim. Biophys. Acta 663 (1981) 255–265.
[26] G. Vassaux, D. Gaillard, G. Ailhaud, R. Negrel, Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP- and Ca(2+)-elevating agent, J. Biol. Chem. 267 (1992) 11092–11097.
[27] G. Vassaux, D. Gaillard, C. Darimont, G. Ailhaud, R. Negrel, Differential response of preadipocytes and adipocytes to prostacyclin and prostaglandin E2: physiological implications, Endocrinology 131 (1992) 2393–2398.
[28] R.K. Petersen, C. Jorgensen, A.C. Rustan, L. Froyland, K. Muller-Decker, G. Furstenberger, R.K. Berge, K. Kristiansen, L. Madsen, Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases, J. Lipid Res. 44 (2003) 2320–2330.
[29] D. Gaillard, R. Negrel, M. Lagarde, G. Ailhaud, Requirement and role of arachidonic acid in the differentiation of pre-adipose cells, Biochem. J. 257 (1989) 389–397.
[30] B.T. Hyman, L.L. Stoll, A.A. Spector, Prostaglandin production by 3T3-L1 cells in culture, Biochim. Biophys. Acta 713 (1982) 375–385.
[31] M. Nagai, K. Tuchiya, H. Kojima, Prostaglandin E_2 increases the calcium concentration in rat brown adipocytes and their consumption of oxygen, Prostaglandins 51 (1996) 377–386.
[32] H. Yan, A. Kermouni, M. Abdel-Hafez, D.C. Lau, Role of cyclooxygenases COX-1 and COX-2 in modulating adipogenesis in 3T3-L1 cells, J. Lipid Res. 44 (2003) 424–429.
[33] T. Yokota, C.S. Meka, K.L. Medina, H. Igarashi, P.C. Comp, M. Takahashi, M. Nishida, K. Oritani, J. Miyagawa, T. Funahashi, Y. Tomiyama, Y. Matsuzawa, P.W. Kincade, Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins, J. Clin. Invest. 109 (2002) 1303–1310.

Downregulation of prostaglandin E receptor subtype EP 3 during colon cancer development

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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 8 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.[14–18] 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.

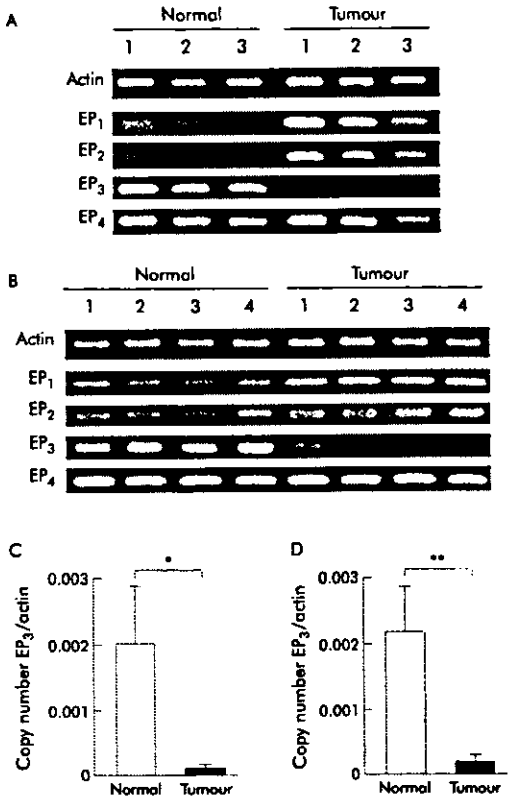

Figure 1 Analyses of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 mRNA expression. (A) Azoxymethane (AOM) treated mouse normal colon mucosa and colon carcinomas. Two pairs of samples (lanes 1, 2) and two independent samples (lane 3) were examine by reverse transcription-polymerase chain 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).

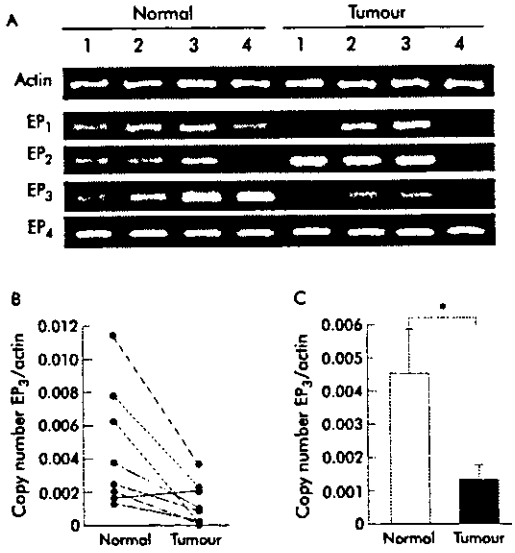

Figure 2 Analyses of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 mRNA expression in human colon tissues. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis patterns in four typical pairs of samples (lanes 1–4) are shown. (B, C) Quantitative real time RT-PCR analysis 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

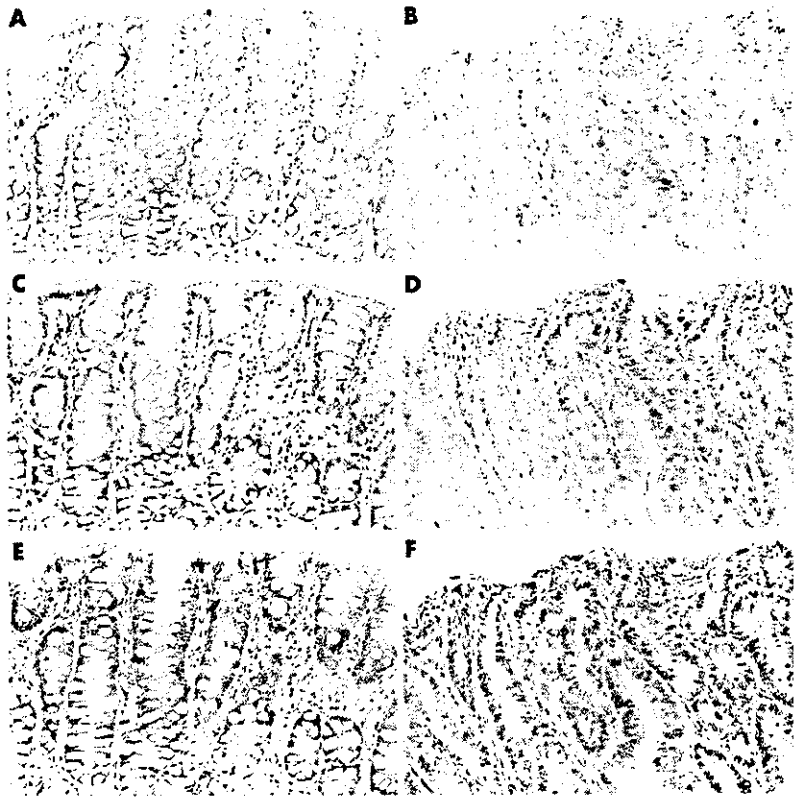

Figure 3 Immunohistochemical staining for the rat prostaglandin E_2 receptor subtype EP_3 of normal colon mucosa (A, C, and E) and colon adenocarcinoma (B, D, and F). Non-specific staining of some red blood cells and weak background staining were observed in the negative controls stained without anti-EP_3 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

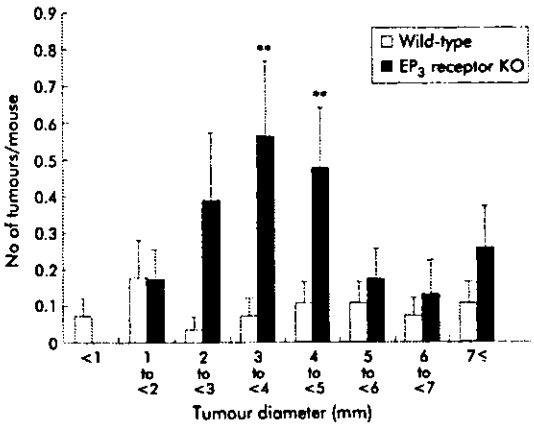

Figure 4 Size distribution of colon tumours induced by azoxymethane in wild-type and prostaglandin E_2 receptor subtype EP_3 knockout (KO) mice. The number of tumours/mouse in each size class is expressed as mean (SEM). **Significantly different from the corresponding wild-type value ($p<0.01$).

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.[25] These reports support our data that down-regulation 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.[26–27] 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.

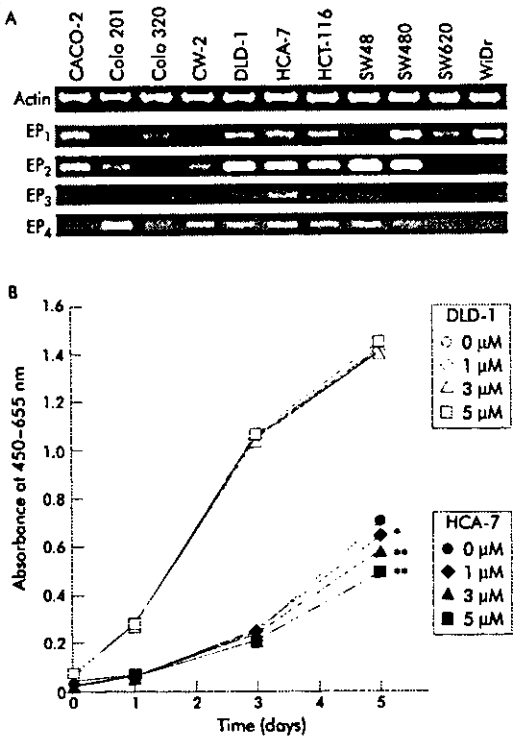

Figure 5 Effect of ONO-AE-248 treatment on cell growth of DLD-1 and HCA-7 cells. (A) Expression of prostaglandin E_2 (PGE_2) receptors EP_1, EP_2, EP_3, and EP_4 was analysed by reverse transcription-polymerase chain reaction in 11 human colon cancer cell lines. (B) DLD-1 and HCA-7 cells were seeded 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 ($n=6$). *$p<0.05$, **$p<0.01$.

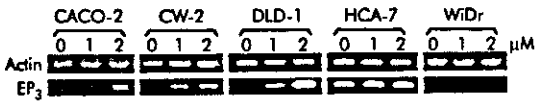

Figure 6 5-Aza-2′-deoxycytidine (5-aza-dC) treatment of CACO-2, CW-2, DLD-1, HCA-7, and WiDr colon cancer cell lines. Each cell line was treated with 1 and 2 μM 5-aza-dC three times. EP_3 receptor expression was analysed by reverse transcription-polymerase chain reaction.

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.[10 11] Two of the three variants of the mouse EP_3 receptors are $EP_{3\gamma}$ and $EP_{3\beta}$, which are coupled to G_i and cause inhibition of adenylate cyclase.[10] 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.[11] 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.[24] In our previous study,[16] 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.[25] 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.

ACKNOWLEDGEMENTS

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REFERENCES

1 **Elder DJE**, Paraskeva C. COX-2 inhibitors for colorectal cancer. *Nat Med* 1998;**4**:392–3.
2 **Reddy BS**, Rao CV, Rivenson A, *et al*. Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. *Carcinogenesis* 1993;**14**:1493–7.
3 **Rao CV**, Rivenson A, Simi B, *et al*. Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Res* 1995;**55**:1464–72.
4 **Fukutake M**, Nakatsugi S, Isoi T, *et al*. Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis* 1998;**19**:1939–42.
5 **Labyte D**, Fischer D, Vieth P, *et al*. Sulindac causes regression of rectal polyp in familial adenomatous polyposis. *Gastroenterology* 1991;**101**:635–9.
6 **Giardiello FM**, Hamilton SR, Krush AJ, *et al*. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;**328**:1313–16.
7 **Hirata K**, Itoh H, Ohsato K. Regression of rectal polyps by indomethacin suppository in familial adenomatous polyposis. Report of two cases. *Dis Colon Rectum* 1994;**37**:943–6.
8 **Hirota C**, Iida M, Aoyagi K, *et al*. Effect of indomethacin suppositories on rectal polyposis in patients with familial adenomatous polyposis. *Cancer* 1996;**78**:1660–5.
9 **Eberhart EC**, Dubois NR. Eicosanoids and the gastrointestinal tract. *Gastroenterology* 1995;**109**:285–301.
10 **Kitamura T**, Kawamori T, Uchiya N, *et al*. Inhibitory effects of mofezolac, a cyclooxygenase-1 selective inhibitor, on intestinal carcinogenesis. *Carcinogenesis* 2002;**23**:1463–6.
11 **Narumiya S**, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. *Physiol Reviews* 1999;**79**:1193–226.
12 **Hirai H**, Tanaka K, Yoshie O, *et al*. Prostaglandin D_2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001;**93**:255–61.
13 **Yang VW**, Shields JM, Hamilton SR, *et al*. Size-dependent increase in prostanoid levels in adenomas of patients with familial adenomatous polyposis. *Cancer Res* 1998;**58**:1750–3.
14 **Rigas B**, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 1993;**122**:518–23.
15 **Pugh S**, Thomas GA. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E_2. *Gut* 1994;**35**:675–8.
16 **Hasegawa H**, Negishi M, Katoh H, *et al*. Two isoforms of prostaglandin EP_3 receptor exhibiting constitutive activity and agonist-dependent activity in Rho-mediated stress fiber formation. *Biochem Biophys Res Commun* 1997;**234**:631–6.
17 **Watanabe K**, Kawamori T, Nakatsugi S, *et al*. Role of the prostaglandin E receptor subtype EP_1 in colon carcinogenesis. *Cancer Res* 1999;**59**:5093–6.
18 **Mutoh M**, Watanabe K, Kitamura T, *et al*. Involvement of prostaglandin E receptor subtype EP_4 in colon carcinogenesis. *Cancer Res* 2002;**62**:28–32.
19 **Sonoshita M**, Takaku K, Sasaki N, *et al*. Acceleration of intestinal polyposis through prostaglandin receptor EP_2 in Apc^{1716} knockout mice. *Nat Med* 2001;**7**:1048–51.
20 **Takahashi M**, Fukuda K, Ohata T, *et al*. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. *Cancer Res* 1997;**57**:1233–7.
21 **Kirkland SC**. Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Res* 1985;**45**:3790–5.
22 **Nakamura K**, Kaneko T, Yamashita Y, *et al*. Immunocytochemical localization of prostaglandin EP_3 receptor in the rat hypothalamus. *Neurosci Lett* 1999;**260**:117–20.
23 Zacharowski K, Olbrich A, Piper J, *et al* Selective activation of prostanoid EP_3 receptor reduces myocardial infarct size in rodents. *Arterioscler Thromb Vase Biol* 1999;**19**:2141–7.
24 **Kawamori T**, Uchiya N, Sugimura T, *et al*. Enhancement of colon carcinogenesis by prostaglandin E_2 administration. *Carcinogenesis* 2003;**24**:985–90.
25 **Sugimoto Y**, Namba T, Honda A, *et al*. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP_3 subtype. *J Biol Chem* 1992;**267**:6463–6.
26 **Katani M**, Tanaka I, Ogawa Y, *et al*. Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP_3 subtype generated by alternative messenger RNA splicing: Multiple second messenger systems and tissue-specific distributions. *Mol Pharmacol* 1995;**48**:869–79.
27 **Ding M**, Kinoshita Y, Kishi K, *et al*. Distribution of prostaglandin E receptors in the rat gastrointestinal tract. *Prostaglandins* 1997;**53**:199–216.
28 **Katani M**, Tanaka I, Ogawa Y, *et al*. Structural organization of the human prostaglandin EP_3 receptor subtype gene (PTGER3). *Genomics* 1997;**40**:425–34.
29 **Goureau O**, Tanfin Z, Marc S, *et al*. Diverse prostaglandin receptors activate distinct signal transduction pathways in rat myometrium. *Am J Physiol* 1992;**263**:C257–65.

30 **Sugimoto Y**, Negishi M, Hayashi Y, *et al*. Two isoforms of the EP_3 receptor with different carboxyl-terminal domains. *J Biol Chem* 1993;**268**:2712–18.
31 **Irie A**, Sugimoto Y, Namba T, *et al*. Third isoform of the prostaglandin-E-receptor EP_3 subtype with different c-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *Eur J Biochem* 1993;**217**:313–18.
32 **Takeuchi K**, Takahashi N, Abe T, *et al*. Functional difference between two isoforms of rat kidney prostaglandin receptor EP_3 subtype. *Biochem Biophys Res Commun* 1994;**203**:1897–903.
33 **Neuschafer-Rube F**, DeVries C, Honecke K, *et al*. Molecular cloning and expression of a prostaglandin EP_3 beta subtype from rat hepatocytes. *FEBS Lett* 1994;**351**:119–22.
34 **Dhanasekaran N**, Tsim ST, Dermott JM, *et al*. Regulation of cell proliferation by G proteins. *Oncogene* 1998;**17**:1383–94.
35 **Baylin SB**, Herman JD. DNA hypermethylation in tumorigenesis: epigenetic joins genetics. *Trends Genet* 2000;**16**:168–74.
36 **Jones PA**, Laird PW. Cancer epigenetics comes of age. *Nat Genet* 1999;**21**:163–7.
37 **Esteller M**, Sparks A, Toyota M, *et al*. Analysis of adenomatous polyposis coli hypermethylation in human cancer. *Cancer Res* 2000;**60**:4366–71.
38 **Toyota M**, Shen L, Ohe-Toyota M, *et al*. Aberrant methylation of the cyclo-oxygenase 2 CpG island in colorectal tumors. *Cancer Res* 2000;**60**:4044–8.
39 **Okuda-Ashitaka E**, Sakamoto K, Ezashi T, *et al*. Suppression of prostaglandin E receptor signaling by the variant from of EP_1 subtype. *J Biol Chem* 1996;**271**:31255–61.

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.

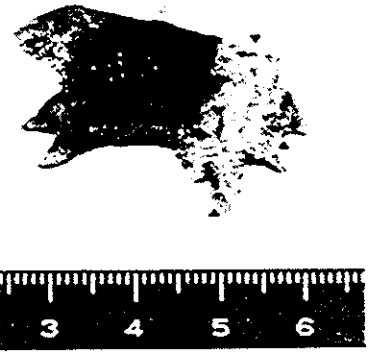

Figure 2 A picture of the foot of a soft shelled turtle.

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Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice

Eiji Nakamura, Takashi Kataoka, Kazuharu Furutani, Keisuke Jimbo, Takeshi Aihara, Satoshi Tanaka, Atsushi Ichikawa, Hiroshi Ohtsu and Susumu Okabe

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Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice

Eiji Nakamura,[1] Takashi Kataoka,[1] Kazuharu Furutani,[1] Keisuke Jimbo,[1] Takeshi Aihara,[1] Satoshi Tanaka,[2] Atsushi Ichikawa,[2] Hiroshi Ohtsu,[3] and Susumu Okabe[1]

[1]*Department of Applied Pharmacology, Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto, 607-8414;* [2]*Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Yoshida, Sakyo-ku, Kyoto, 606-8501; and* [3]*Department of Cellular Pharmacology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai, Japan 980-8575*

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Nakamura, Eiji, Takashi Kataoka, Kazuharu Furutani, Keisuke Jimbo, Takeshi Aihara, Satoshi Tanaka, Atsushi Ichikawa, Hiroshi Ohtsu, and Susumu Okabe. Lack of histamine alters gastric mucosal morphology: comparison of histidine decarboxylase-deficient and mast cell-deficient mice. *Am J Physiol Gastrointest Liver Physiol* 287: G1053–G1061, 2004. First published July 22, 2004; doi:10.1152/ajpgi.00353.2003.—Histamine plays an important role in the regulation of gastric acid secretion; however, its role in maintenance of gastric morphology remains unclear. To clarify the necessity of histamine for gastric mucosal development and maintenance, we evaluated two different kinds of mice that lacked either mast cells (one of the gastric histamine-producing cell types) or histidine decarboxylase (HDC; a histamine-synthesizing enzyme). Measurements of stomach weight, intragastric pH, mucosal histamine levels, as well as serum gastrin and albumin levels were performed in mice. Gastric mucosal appearance was examined by immunohistochemical techniques. Although gastric mucosal histamine levels in mast cell-deficient mice were half of those observed in the wild-type mice, intragastric pH, serum gastrin levels, and gastric morphology at 12 mo were unchanged compared with the wild-type mice. In contrast, HDC-deficient mice possessed no detectable gastric histamine, but did exhibit hypergastrinemia, as well as marked increases in intragastric pH and stomach weight compared with the wild-type mice. Histological analysis revealed that 9-mo-old HDC-deficient mice demonstrated hyperplasia in the oxyntic glandular base region, as well as increased numbers of parietal and enterochromaffin-like cells. These results indicate that enterochromaffin-like cell-derived histamine is potentially involved in gastric mucosal morphology regulation.

enterochromaffin-like cell; parietal cell; hypergastrinemia

GASTRIC MUCOSA CONSISTS OF numerous blind tubular units containing various cell types (28). All gastric mucosal epithelial cells are known to originate from common progenitor cells in the proliferative zone of the isthmus. Some cells migrate upward, becoming mucous-secreting surface epithelial cells, whereas other cells migrate toward the base of the gland, differentiating into parietal, chief, or endocrine cells (18). Maintenance of gastric mucosal integrity and structure is regulated by a variety of endocrine- and paracrine-mediating factors.

Histamine is synthesized from histidine by histidine decarboxylase (HDC) and is stored in mast cells, enterochromaffin-like (ECL) cells, and enteric nerve fibers in the oxyntic mucosa of rodent stomachs (25, 46). It is well established that histamine plays a pivotal role in gastric acid secretion (1, 47); however, its role in the regulation of gastric mucosal proliferation and differentiation essentially remains uncharacterized. To clarify such a role, previous reports (16, 39, 40) have used pharmacological inhibitors of histamine pathways, such as histamine receptor (H_1R, H_2R, and H_3R) antagonists and α-fluoromethylhistidine (α-FMH), an irreversible inhibitor of HDC (4, 5, 10). Nonetheless, incomplete elimination of histamine action by these inhibitors could potentially confuse analysis of the exact role of histamine for regulation of gastric mucosal morphology.

Recently, HDC-deficient (HDC-KO) mice have been generated by gene-targeting methods (45, 52). As expected, these mice exhibited no de novo gastric mucosal histamine synthesis (52). Using 8- to 12-wk-old HDC-KO mice, we reported that gastric mucosal morphology was similar to the wild-type (WT) mice (52), although HDC-KO mice showed hypoacidity and hypergastrinemia (24, 52). In this study, we examined the role of histamine in the regulation of gastric mucosal morphology in HDC-KO mice longer term, from 1 to 9 mo. In addition, the present study characterized the specific role of ECL cell-derived histamine by comparing the difference between HDC-KO mice and mast cell-deficient mice.

MATERIALS AND METHODS

Animals. Six-week-old male mast cell-deficient $WBB6F_1$-*W/W*^{v} (*W/W*^{v}) and congenic normal $WBB6F_1$-+/+ (+/+) mice were purchased (Japan SLC, Shizuoka, Japan). The two mice strains were independently maintained until an age of 3 or 12 mo. Male HDC-KO as well as the WT littermate mice were generated on a mixed genetic 129/Sv times ICR background, and raised with regular diet, and independently maintained until an age of 1, 3, 6, or 9 mo (24, 45, 52). All of the mice were given standard pellets (CE-2; CLEA, Tokyo, Japan). Mice were deprived of food for 21 h and water for 2 h before each experiment. Plasma and tissue samples from pairs of age-matched WT and mutant mice were compared. Animal maintenance and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of Kyoto Pharmaceutical University.

Measurement of serum gastrin and albumin levels, as well as intragastric pH. Blood collected from mice was centrifuged at 6,000 *g* for 15 min to obtain serum samples. Serum gastrin levels were determined by a radioimmunoassay (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan) and expressed as picograms of gastrin per

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milliliter of serum. Serum albumin levels were determined by a bromcresol green assay (6, 21) and expressed as grams of albumin per deciliter of serum. Stomachs removed from mice were incised along the greater curvature, and intragastric pH was measured by directly placing a pH meter on the fundic mucosa.

Determination of gastric mucosal histamine levels. Each sample for histamine quantification was collected according to a previously reported method (29). In brief, each stomach was rinsed with PBS containing 10^{-6} M semicarbazide hydrochloride, weighed, and homogenized in 0.01 M PBS. The homogenates were diluted 1:10 with phosphate buffer and heated in boiling water for 10 min to release bound histamine. The homogenates were then centrifuged at 3,300 *g* for 20 min, and the resulting supernatants were used for measurements. Quantification of sample histamine levels was performed with a histamine enzyme immunoassay kit (Immunotech, Marseilles, France).

Measurement of gastric mucosal protein and DNA levels. To measure gastric mucosal protein levels, the entire glandular stomach of each mouse was homogenized with 1 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 1 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 0.1% aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The homogenates were centrifuged at 10,000 *g* for 30 min at 4°C. The protein levels for each supernatant were measured with a protein quantification kit (Bio-Rad, Hercules, CA) and expressed as milligrams of protein per stomach. To quantify DNA levels, high molecular weight DNA was first isolated by digestion of the stomach with proteinase K, followed by phenol-chloroform extraction. The concentration of DNA solubilized in water was then quantified from the absorbance at 260 nm and the DNA levels in gastric mucosa was calculated and expressed as micrograms of DNA per stomach. The ratios of the protein levels or the DNA levels between HDC-KO and WT mice (KO/WT ratio) were calculated and compared each ratio to evaluate whether it is hyperplasia (increased DNA levels) or hypertrophy (increased cell volume, but not DNA levels).

Treatment. To evaluate the role played by hypergastrinemia in gastric oxyntic mucosal hyperplasia induced in HDC-KO mice, (R)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) (YM022) urea from Yamanouchi Pharmaceutical (Tokyo, Japan) (31, 41) was administered orally for 2 mo to 1-mo-old HDC-KO mice. YM022 represents a selective cholecystokinin type 2 receptor (CCK_2R) antagonist; a dose of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ was used, because this dose inhibited gastrin-17-stimulated gastric acid secretion in mice by >80% in our preliminary experiments. Omeprazole was administered orally at a dose of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 mo to 1-mo-old WT mice to induce hypergastrinemia and allow comparison with HDC-KO mice in terms of hyperplasia. Both drugs were suspended in 0.5% hydroxypropylcellulose solution and were administered once daily at a volume of 5 ml/kg body wt.

Histochemical analysis. For hematoxylin and eosin staining, as well as immunonhistochemical analysis, of the gastric mucosa, the stomach samples were fixed with Carnoy's fixative overnight, embedded in paraffin wax, and then sectioned at a slice thickness of 4 μm. Paraffin sections stained with hematoxylin and eosin or periodic acid Schiff were used for determination of gastric mucosal thickness. Under a microscope, well-orientated regions spanning from the gastric base to the oxyntic mucosal surface were selected for measurement. The mucosal thickness was taken as the average of measurements in four different visual fields for each stomach with the use of a calibrated-eyepiece micrometer scale. Immunohistochemical analysis of parietal and endocrine cells was performed for each section with the use of the following avidin-biotin-peroxidase immunohistochemical technique. Parietal, D, and endocrine cells were detected with a murine monoclonal antibody for α-subunit of murine H^+-K^+-ATPase (Medical and Biological Laboratories, Nagoya, Japan), a rabbit antibody for somatostatin (DAKO, Carpinteria, CA), and a rabbit antibody for a peptide (359–389 amino acid) of rat chromogranin A (Cg A; Yanaihara Institute, Fujinomiya, Japan), respectively. In brief, endogenous peroxidase activity was blocked with a methanol solution containing 0.3% hydrogen peroxide. Sections were incubated with primary antibody for α subunit of H^+-K^+-ATPase or Cg A for 2 h at room temperature. Sections were then incubated with secondary biotinylated antibodies, followed by streptoavidin peroxidase. Finally, slides were developed with diaminobenzidine and counterstained with hematoxylin. Parietal and endocrine cell counts were quantified in samples in which mucosal glands were perpendicularly oriented to the mucosal surface. Cell counts for parietal cells positively immunoreactive for H^+-K^+-ATPase were determined in four different gland units for each stomach; results were expressed as the number of cells per gland unit. Cell counts for endocrine cells positively immunoreactive for Cg A or D cells positively reactive for somatostatin were determined in four different visual fields for each stomach; results were expressed as the average number of cells per visual field (0.25 mm^2). An average for each group of animals was also calculated.

Statistics. Data are expressed as means ± SE. Statistical differences were evaluated by using the Student's *t*-test and Dunnett's multiple comparison test, with a P value < 0.05 regarded as significant. Dunnett's test was performed after a significant ANOVA had been achieved.

RESULTS

Mast cell deficiency exerted no effect on gastric morphology. Deletion of mast cells (W/W^v) caused a significant decrease in gastric mucosal histamine levels by ~50% compared with +/+ mice (Fig. 1*A*). However, the reduction in histamine levels did not affect intragastric pH (Fig. 1*B*) or serum gastrin levels (Fig. 1*C*), which were found to be similar for both +/+ and W/W^v mice. In addition, histological analysis revealed that W/W^v mice failed to exhibit any morphological alterations in the gastric oxyntic mucosa, even 3 and 12 mo after birth (Fig. 1, *D–G*). Ulcer was observed only in the gastric antrum of W/W^v mice (data not shown), corresponding with the previous report by Shimada et al. (49).

Long-term histamine deficiency resulted in gastric mucosal hyperplasia, hypochlorydria, and hypergastrinemia in HDC-KO mice. HDC-KO mice developed without any obvious abnormality in general appearance, exhibiting body weight gain similar to WT mice for ≤9 mo after birth. As expected, HDC-KO mice possessed undetectable histamine in the stomach (Fig. 2*A*). It was of interest that intragastric pH in HDC-KO mice was significantly higher than that measured in WT mice at all time points examined (1, 3, 6, and 9 mo after birth; Fig. 2*B*). In addition, HDC-KO mice exhibited a marked age-dependent gastric weight gain compared with WT mice, which was first obvious 3 mo after birth (Fig. 2*C*). Moreover, HDC-KO mice serum gastrin levels remained markedly elevated at all time points examined (Fig. 2*D*). Serum albumin levels were similar for WT and HDC-KO mice 6 and 9 mo after birth (Fig. 2*E*). No correlation was observed between body weight and stomach weight in either the WT or HDC-KO mice.

To thoroughly ascertain the increased stomach weight observed in HDC-KO mice, gastric mucosal protein and DNA levels were compared for 6-mo-old WT and HDC-KO mice. Protein levels in HDC-KO mice were significantly higher than those of WT mice (17.47 ± 1.89 mg/stomach vs. 11.80 ± 0.74 mg/stomach; KO/WT, 1.48). DNA levels in HDC-KO mice were approximately two times higher than those of WT mice