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# Expression of L-histidine decarboxylase in granules of elicited mouse polymorphonuclear leukocytes

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Infiltrating polymorphonuclear leukocytes (PMN) in the peritoneal cavity were found to express L-histidine decarboxylase (HDC), the rate-limiting enzyme of histamine synthesis, in a csein-induced peritonitis model. Expression of HDC was detected in the elicited PMN, but not in the peripheral blood leukocytes. The peritoneal lavage fluids in this model were found to augment histamine synthesis in PMN isolated from the bone marrow. Rapid post-translational processing of HDC was observed in PMN, and the dominant form of HDC was the mature 53-kDa form, which was found to co-localize with a granule enzyme, matrix metalloproteinase-9 (MMP-9). Treatment of PMN with the phorbol ester PMA, which stimulates the release of MMP-9, did not liberate the granular HDC. Immunofluorescence studies using an anti-HDC antibody strongly suggested that HDC is bound to the cytosolic side of the granule membranes. These observations suggest that HDC is induced upon infiltration of PMN into the mouse peritoneal cavity and that histamine is synthesized by HDC attached to the granule membranes of PMN.

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## 1 Introduction

Histamine has been reported to play various roles in the modulation of immune and inflammatory responses via the $H_1$ and $H_2$ receptors [1], such as increase in vascular permeability [2], leukocyte rolling [3], cytokine production [4, 5], polarization of dendritic cells [6], and T cell and antibody responses [7, 8]. These studies suggest that endogenous histamine production can modulate immune responses, although the exact source of this histamine remains to be fully determined. Many of these studies have assumed that the histamine released from mast cells and basophils via degranulation is important in such kinds of immune regulation. However, since this histamine release is generally observed at the onset of inflammatory responses, it is unlikely that only histamine released from mast cells and basophils can modulate the immune responses, especially during the late phase responses. In an air pouch-type allergic inflammation model in rats, the release of histamine into the pouch fluid was found to be biphasic [9]: in the anaphylactic phase, activated mast cells in the pouch released histamine, causing a transient increase in vascular permeability via the $H_1$ receptors [2], and in the following chronic phase, cells of unknown identity, which were not mast cells, produced histamine, causing the inhibition of leukocyte infiltration into the pouch via the $H_2$ receptors [10]. During the chronic phase of inflammation, an intensive infiltration of polymorphonuclear leukocytes (PMN) and macrophages is usually observed. A detectable amount of histamine synthesis was found to be induced in the murine myelomonocytic cell line WEHI-3B, in parallel with their differentiation into macrophages [11]. Mirossay et al. have demonstrated the existence of trace amounts of L-histidine decarboxylase (HDC) activity and histamine content in macrophages as well as in the human promyelocytic leukemia cell line HL-60, which can be differentiated into granulocytes by all-*trans*-retinoic acid treatment [12]. These *in vitro* studies suggest that both PMN and macrophages may be involved in histamine synthesis during the chronic phase of the inflammatory response. Although it still remains unknown as to which cell type is responsible for histamine synthesis *in vivo*, it is important to clarify the regulation of histamine synthesis by these cell types in order to appreciate the modulatory role of histamine in immune and inflammatory responses.

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**Abbreviations: BM-PMN:** PMN precursors from bone marrow **ER:** Endoplasmic reticulum **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase **HDC:** L-Histidine decarboxylase **MMP-9:** Matrix metalloproteinase-9 **PDI:** Protein disulfide isomerase **SLO:** streptolysin-O

We previously described the post-translational processing and intracellular localization of HDC (EC 4.1.1.22), the rate-limiting enzyme in histamine synthesis in mammals, in the rat basophilic/mast cell line RBL-2H3 [13]. HDC was found to be translated as a 74-kDa precursor form in the cytosol, which is then processed into its mature 53-kDa form. In addition, histamine synthesis was detected in two distinct compartments, in the cytosol and in granules. On the other hand, in the preliminary study, we have found that PMN are the dominant cell type responsible for histamine synthesis in an experimental casein-induced peritonitis model [14]. Granules in human PMN have been classified into three types; azurophil/primary, specific/secondary, and gelatinase/tertiary granules [15], whereas such a classification of neutrophil granules remains to be fully determined in mice. Although the previous observation that azurophil granules in PMN share several characteristics with histamine-containing granules in mast cells [16] indicates the granular localization of HDC in PMN, the exact intracellular localization of HDC in PMN remains to be clarified. In this study, we investigated the post-translational processing and intracellular localization of HDC to elucidate the regulation of histamine synthesis in PMN.

## 2 Results

### 2.1 Expression of HDC in PMN

In our preliminary study, we have found that PMN are responsible for histamine synthesis in an experimental casein-induced peritonitis model [14]. In this model, a drastic and transient increase in HDC enzyme activity in total peritoneal cells was observed, reaching a peak level 5 h after casein injection, at which time an intensive infiltration of PMN into the peritoneal cavity was observed ([14] and data not shown). PMN were purified from the peritoneal cells 5 h after casein injection. Immunofluorescence studies revealed that the purified PMN fraction (>98% purity, confirmed by May-Grünwald-Giemsa staining) was immunoreactive to both an anti-HDC and an anti-CD11b antibody (Fig. 1A). The immunofluorescence pattern indicated a granule-like localization of HDC in the PMN. On the other hand, HDC$^+$ cells were not found in total leukocytes from peripheral blood, both in control mice and in mice injected with casein, although a significant number of CD11b$^+$ cells were observed in peripheral blood leukocytes from both mice (Fig. 1B). In addition, no detectable amount of HDC activity could be measured in the homogenates of peripheral blood leukocytes collected from mice treated with or without casein for 5 h (data not shown). These results indicate that HDC may be induced in response to inflammatory stimuli in

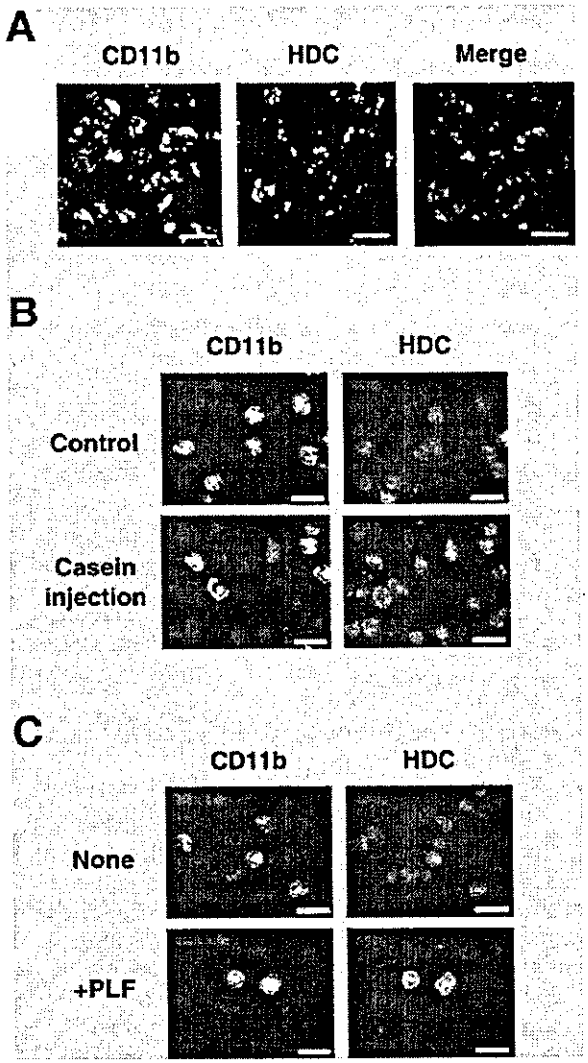


*Fig. 1.* Expression of HDC in infiltrated PMN, and not in peripheral blood leukocytes, in the casein-induced peritonitis model. (A) PMN were purified from peritoneal cells 5 h after casein injection. Cells were centrifuged onto slide glasses and immunofluorescence studies were performed with an anti-HDC antibody (1:500) followed by incubation with an Alexa594-conjugated anti-rabbit IgG antibody (1:1,000; HDC) and a FITC-conjugated anti-CD11b antibody (1:1,000; CD11b). Bars =20 μm. (B) Peripheral blood was collected from control mice and mice 5 h after casein injection, and the leukocyte fraction was purified. HDC-immunoreactive cells were never observed in any of the multiple microscopic fields analyzed. Bars =20 μm. (C) Peripheral blood leukocytes were collected from mice 5 h after casein injection. Total leukocytes were incubated for 6 h under standard culture conditions in the presence (PLF) or absence (None) of the peritoneal lavage fluid obtained 5 h after the injection. Bars =20 μm.

 

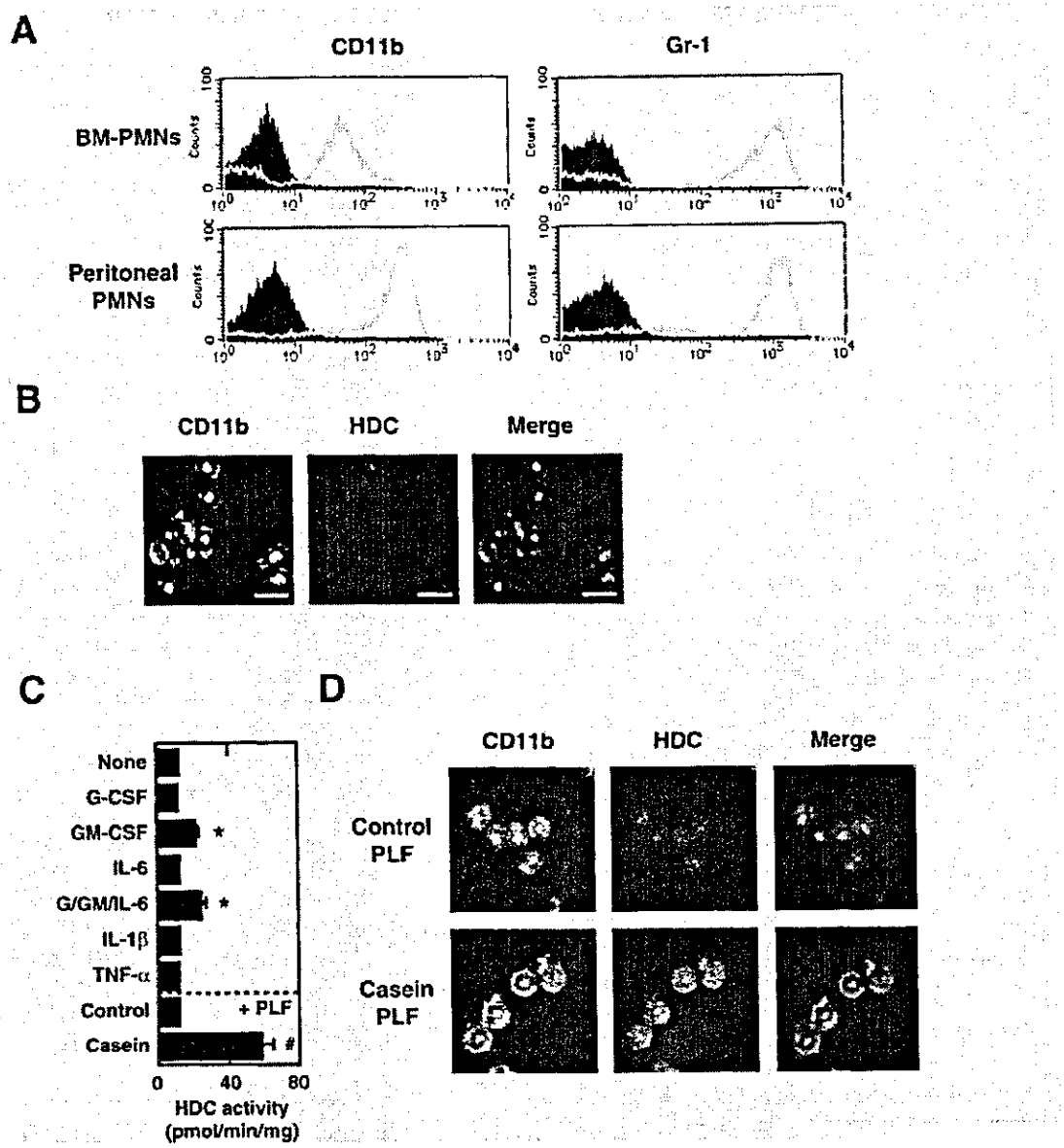


*Fig. 2.* Induction of HDC in BM-PMN. (A) PMN were isolated from bone marrow by density gradient fractionation (BM-PMN). The lineage of the cells was confirmed by flow cytometry with an anti-CD11b antibody and an anti-Gr-1 antibody. The closed area profiles were obtained with the isotype control antibody. (B) BM-PMN were subjected to immunofluorescence study as described in the legend to Fig. 1. Bars =20 μm. (C) BM-PMN were incubated for 3 h in the absence (None) or presence of the indicated cytokines (20 ng/ml), the peritoneal lavage fluids obtained from mice injected with casein (Casein PLF) or those from control mice (Control PLF). Cells were harvested and HDC activity was measured. Values are represented as the means ± SEM ($n$=3–6). $^{*}p<0.05$ is regarded as significant by the Student's $t$ test (*vs.* None). $^{\#}p<0.05$ is regarded as significant by the Student's $t$ test (*vs.* Control PLF). (D) BM-PMN were incubated for 3 h in the presence of the peritoneal lavage fluids obtained from mice injected with casein (Casein PLF) or from control mice (Control PLF). Incubated BM-PMN were subjected to immunofluorescence study as described in the legend to Fig. 1. Bars =20 μm.

the cavity. To confirm this hypothesis, we collected peripheral blood leukocytes and peritoneal lavage fluid from peritoneal cavities 5 h after casein injection. After incubation of the peripheral blood leukocytes with the lavage fluid for 6 h at 37°C, a significant number of HDC$^{+}$ cells could be detected (Fig. 1C).

 

### 2.2 Induction of HDC in PMN isolated from bone marrow

Since a method for purification of PMN from murine peripheral blood leukocytes remains to be established, we isolated PMN precursors from the bone marrow (BM-PMN) by density gradient fractionation. Flow cytometric analysis demonstrated that greater than 80% of this fraction was immunoreactive to both an anti-CD11b and an anti-Gr-1 antibody (Fig. 2A). In comparison with casein-induced peritoneal PMN, similar levels of surface expression of Gr-1 and lower levels of CD11b were observed in BM-PMN. Immunofluorescence studies revealed that $CD11b^+$ cells in BM-PMN were not immunoreactive to the anti-HDC antibody (Fig. 2B). We then investigated effects of peritoneal lavage fluids and several cytokines on the induction of histamine synthesis in BM-PMN. A drastic increase in HDC activity was observed in BM-PMN incubated for 3 h in the presence of peritoneal lavage fluids collected from mice injected with casein, but not in the presence of fluids collected from control mice (Fig. 2C). Induction of HDC by the lavage fluids was also confirmed by immunofluorescence study with the anti-HDC antibody (Fig. 2D). Among the cytokines tested, treatment with GM-CSF was found to slightly but significantly augment HDC activity in BM-PMN (Fig. 2C). IL-1β, IL-6, TNF-α, and G-CSF did not induce HDC activity in BM-PMN.

### 2.3 Expression and enzymatic activity of HDC in PMN

We then investigated the expression of mRNA and protein and the enzymatic activity of HDC in peritoneal PMN. Northern blot analyses demonstrated a high level of HDC mRNA expression in PMN immediately after purification, followed by a rapid decrease under culture conditions (Fig. 3A). Immunoblot analysis using the anti-HDC antibody revealed that the dominant molecular species in peritoneal PMN is the 53-kDa form (Fig. 3B). Purified PMN showed a significant level of HDC activity (~80 pmol/min/mg protein), which gradually decreased under standard culture conditions for up to 6 h (Fig. 3C). Post-translational processing of HDC was confirmed by pulse-chase experiments (Fig. 3D, E). The 74-kDa precursor form was found to be completely converted into its mature 53-kDa form within 1 h. A band of approximately 120 kDa with strong labeling was detected, but was still detectable in the presence of excess amounts of the antigen, glutathione-S-transferase HDC fusion protein (data not shown), and hence we conclude that this band is from an unrelated protein detected in our system.

### 2.4 Co-localization of HDC with MMP-9

We investigated the intracellular localization of HDC. PMN were double-stained with the anti-HDC antibody and an antibody against matrix metalloproteinase-9 (MMP-9, gelatinase B), which is known to be a granule proteinase in PMN. In confocal microscopic observations, HDC and MMP-9 were found to co-localize in the granules of PMN (Fig. 4A), indicating that HDC is localized in the MMP-9-containing granules of PMN.

### 2.5 Unchanged localization of HDC upon stimulation with PMA

Since previous studies have demonstrated that MMP-9 is liberated from PMN upon treatment with phorbol 12-myristate 13-acetate (PMA) [17], extracellular release of HDC was investigated. A dose-dependent release of MMP-9 was measured as both enzyme activity and immunoreactive protein upon treatment of PMN with PMA for 30 min (Fig. 4B), whereas no release of HDC enzyme activity (data not shown) and immunoreactive protein was detected under the same conditions (Fig. 4B). The absence of MMP-9 and the granule-associated expression of HDC upon PMA treatment were also confirmed by immunofluorescence studies (Fig. 4C). Although relatively high levels of spontaneous histamine release (approximately 20%) were detected, a significant amount of stimulated release was detected upon treatment with PMA (Table 1). On the other hand, neither *N*-formyl-methionyl-leucyl-phenylalanine (up to 10 μM) nor leukotriene $B_4$ (up to 1 μM) was found to induce a significant histamine release (data not shown).

### 2.6 Membrane orientation of HDC

We then investigated in detail the localization of HDC in the granules of PMN using streptolysin-O (SLO), which selectively permeabilizes plasma membranes [18]. Neither HDC activity nor a band immunoreactive to the anti-HDC antibody was detected in the cytosol fractions obtained after SLO treatment, whereas more than 80% of lactate dehydrogenase activity was recovered (data not shown), excluding the possibility that HDC is a cytosol-soluble protein. Immunoreactive signals were observed with the anti-HDC antibody, and not with the anti-MMP-9 antibody, in the SLO-treated PMN (Fig. 5), indicating that HDC was accessible to the antibody entering from the cytosolic side whereas MMP-9 was not. When the SLO-treated cells, after fixation, were further permeabilized with 1% Triton X-100, co-localization of HDC and MMP-9 was again confirmed.

 

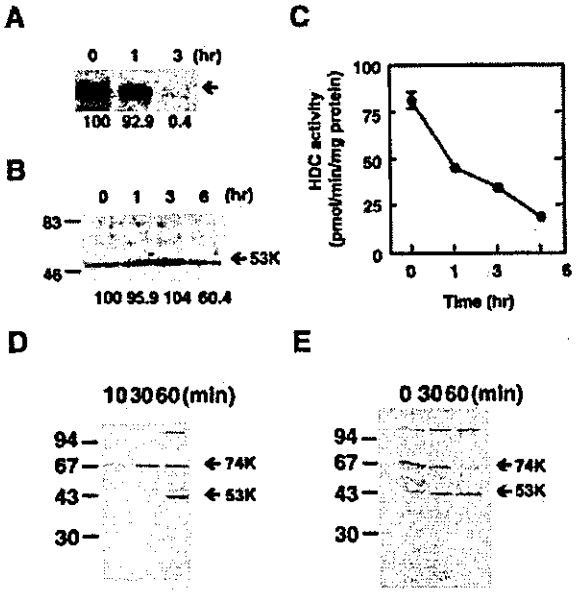


*Fig. 3.* Expression and post-translational processing of HDC in peritoneal PMN under standard culture conditions. Peritoneal PMN were incubated under standard culture conditions for the indicated periods. (A) Northern blot analyses were performed using a $^{32}$P-labeled probe for HDC (total RNA, 3 μg/lane). The relative intensity of each band was normalized using the probe for GAPDH and is presented below the panel.(B) Immunoblot analysis was performed using the anti-HDC antibody (1:500). The arrow indicates the 53-kDa mature form of HDC (53 K). The relative intensity of each band was measured densitometrically and is presented below the panel. The 74-kDa form of HDC was not detectable. (C) Enzyme activity of HDC was measured and presented as mean ± SEM ($n$=5). (D) PMN ($2\times10^7$ cells/lane) were metabolically labeled with [$^{35}$S]methionine in methionine-free medium for the indicated periods. Cells were immunoprecipitated with the anti-HDC antibody. The immunoprecipitates were separated by SDS-PAGE and subjected to autoradiography. (E) PMN labeled with [$^{35}$S]methionine for 30 min were subjected to chase experiments in the presence of 1 mM methionine for the indicated periods. The arrows in (D) and (E) indicate the precursor form of HDC (74 K) and the mature form of HDC (53 K).

## 3 Discussion

In the previous report [14] and in the current study we demonstrated that isolated PMN express HDC and produce histamine in a casein-induced experimental peritonitis model. Expression of HDC in purified peritoneal PMN was demonstrated at both mRNA and protein levels. These results are consistent with previous findings by Shiraishi et al. [19] that infiltrating leukocytes in an air pouch-type allergic inflammation model and in a casein-induced peritonitis model in rats expressed HDC mRNA.

**Table 1.** Histamine release from PMN stimulated with PMA[a)]

| PMA (nM) | Total (ng/$10^7$ cells) | Release (ng/$10^7$ cells) | Net release (%) |
|---|---|---|---|
| 0 | 275±15.4 | 54.2±13.9 | |
| 10 | 272±39.7 | 79.8±4.41 | 8.58±5.40 |
| 30 | 278±25.8 | 83.9±14.8 | 10.1±4.66 |
| 100 | 287±40.5 | 96.9±19.6 | 13.5±7.52 |

[a)] PMN purified from peritoneal cells 5 h after casein injection were incubated under standard culture conditions in the presence of the indicated concentrations of PMA for 30 min. Culture medium and cells were separated by centrifugation at 2,000×g for 5 min at 4°C. Values for net release were calculated by subtracting the value for spontaneous release (PMA 0 nM; 54.2±13.9 ng/$10^7$ cells). The values are presented as means ± SEM ($n$=5).

Although their results strongly suggest that PMN are the dominant source of *de novo* synthesis of histamine (~1.2 pmol/min/mg protein in infiltrating cells), they did not exclude the possibility that macrophages may produce some of this histamine. We have determined the specific activity of HDC in the peritoneal PMN (~80 pmol/min/mg protein, Fig. 3A), which was much higher than previously reported for activated macrophages and mast cells (<10 pmol/min/mg protein) [10, 20]. Furthermore, it is notable that HDC expression was induced in PMN in the peritoneal cavity, since peripheral blood leukocytes did not express HDC. It has been reported that CD11b expression in intravascular neutrophils can be enhanced by the addition of peritoneal fluid supernatant in a glycogen-induced peritonitis model [21]. In the casein-induced model, we have also found that peritoneal lavage fluid has the potential to induce HDC protein in peripheral blood CD11b$^+$ cells and in PMN isolated from the bone marrow (BM-PMN). Therefore, some humoral factors may exist in the peritoneal cavity that can induce HDC expression in PMN. The rapid decrease of HDC mRNA expression under culture conditions supports this hypothesis. We found an increase in HDC activity in BM-PMN incubated in the presence of GM-CSF. G-CSF and IL-6 demonstrated neither additive nor synergistic effects on GM-CSF-mediated induction of HDC, although these cytokines are also involved in regulation of PMN functions. Since the potential of the peritoneal lavage fluids to augment histamine synthesis was much greater than that of GM-CSF, it is possible that the peritoneal lavage fluids contain other inducing factors for HDC. TNF-α is one of the possible candidates, since Endo has previously reported that a systemic injection of TNF-α was able to induce histamine synthesis in mouse bone marrow cells and

 

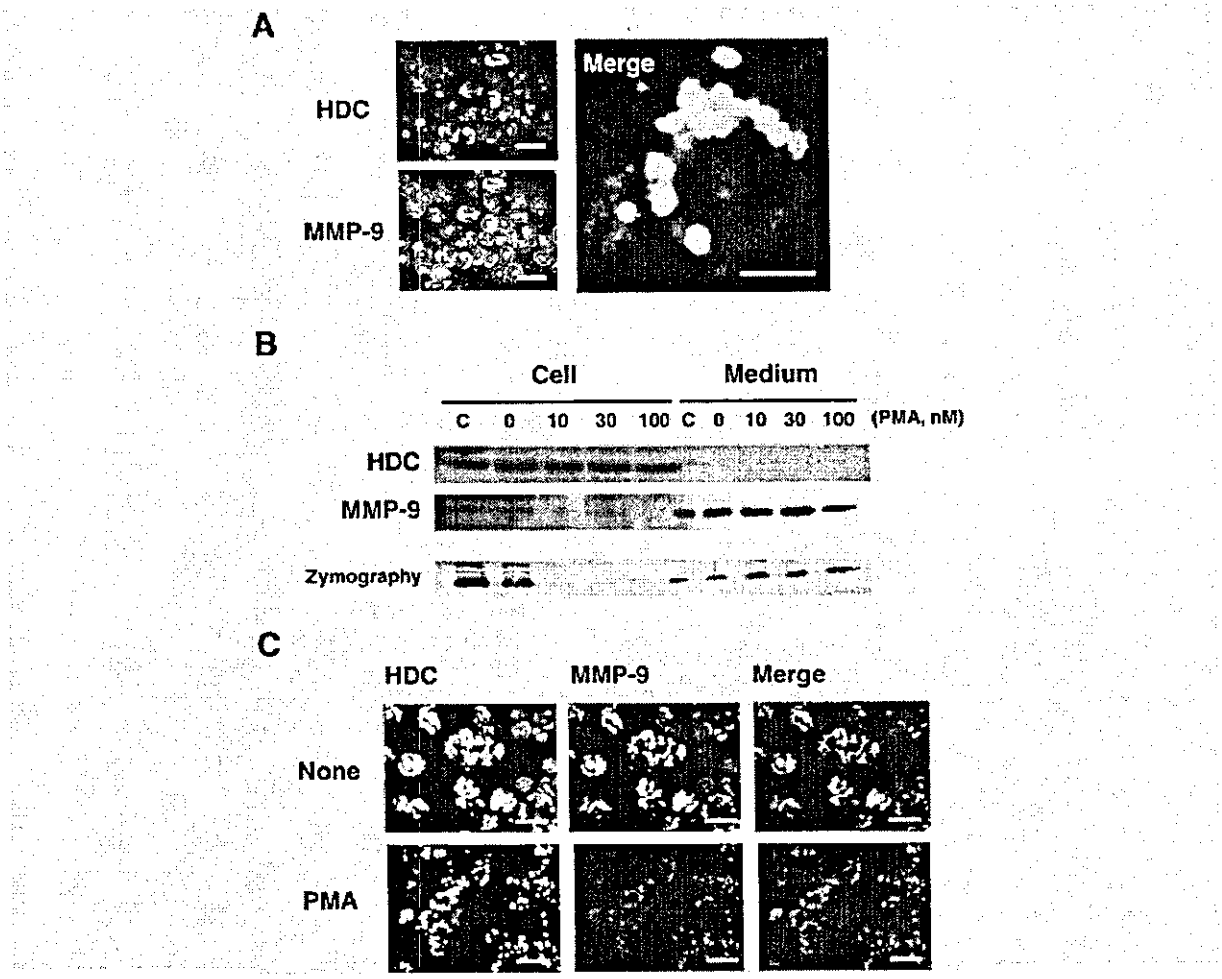


*Fig. 4.* Unchanged localization of HDC in granules upon treatment with PMA. (A) Peritoneal PMN were centrifuged onto slide glasses. Immunofluorescence studies were performed with an anti-HDC antibody (1:500, HDC) and an anti-MMP-9 antibody (1:500, MMP-9) followed by incubation with a FITC-conjugated anti-rabbit IgG antibody (1:100, green) and a rhodamine-conjugated anti-goat IgG antibody (1:100, red). Bars =20 μm. A magnified view is presented (Merge, right panel). Bar =5 μm. (B) PMN were incubated under standard culture conditions in the presence of the indicated concentrations of PMA for 30 min. Control experiments (C) were performed without incubation. Culture medium (Medium) and cells (Cell) were separated by centrifugation at 2,000×*g* for 5 min at 4°C. Each fraction was subjected to immunoblot analyses using the anti-HDC antibody (1:500) and the anti-MMP-9 antibody (1:500). Gelatin zymography was performed as described in section 4.12. (C) PMN were incubated under standard culture conditions in the presence (PMA) or absence (None) of 100 nM PMA for 30 min. Immunofluorescence studies were performed as described above. Bars =10 μm.

spleen cells [22] and since a significant amount of TNF-α release from mast cells has been detected during the anaphylactic phase of a mouse peritonitis model [23]. However, TNF-α may not be involved in the induction of HDC in our model, as TNF-α did not augment HDC activity in BM-PMN and an intraperitoneal injection of an anti-TNF-α neutralizing antibody did not inhibit the induction of HDC (data not shown). Another proinflammatory cytokine, IL-1β, was also found to have no inducible effects on HDC expression in PMN. Further studies are surely required to determine the factors responsible for induction of HDC.

The enzymatic activity of HDC in peritoneal PMN was found to rapidly decrease under culture conditions, before the decrease in HDC mRNA and protein. This result indicates the occurrence of enzymatical inactivation of HDC in peritoneal PMN in addition to degradation of its mRNA and protein, although such inactivation has not been previously reported. Rapid suppression of his-

 

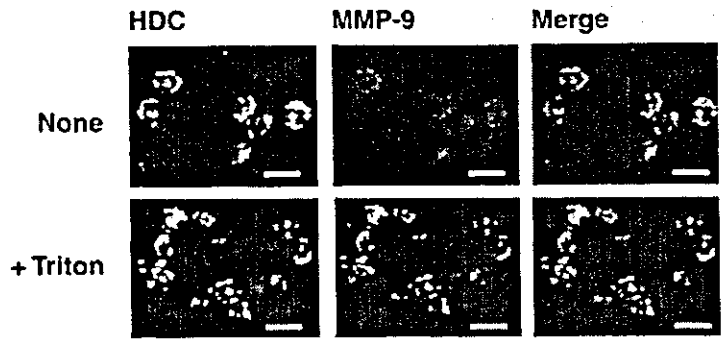


*Fig. 5.* Granular membrane orientation of HDC. PMN were treated with SLO (12,500 U/ml) and fixed. Cells were further permeabilized with (+ Triton) or without (None) 1% Triton X-100 and then stained using the anti-HDC antibody or the anti-MMP-9 antibody as described in the legend to Fig. 3. Bars =10 μm.

tamine synthesis in peritoneal PMN may contribute to the prevention of exacerbated inflammatory responses, which could be induced by prolonged histamine release.

The immunofluorescence studies using the anti-HDC antibody revealed a granular localization of HDC in PMN. We previously demonstrated that HDC was translated in the cytosol and targeted to the endoplasmatic reticulum (ER), where post-translational processing occurred in the rat mast cell line RBL-2H3 [13]. We also observed that histamine was produced in both the cytosol and the granule fractions of the cells, mediated by the 74-kDa form and the 53-kDa form, respectively. Compared with the intracellular localization of HDC in the mast cell line, which is mainly in the ER, the current study demonstrated that HDC is co-localized with MMP-9 in PMN, indicating the granular localization of HDC. These results indicate that HDC may be efficiently processed into the mature 53-kDa form and transported to the gelatinase-containing granules in PMN. Indeed, the pulse-chase experiments showed the rapid and complete processing of HDC as only the 53-kDa form was detected upon immunoblot analyses using the anti-HDC antibody.

We previously demonstrated that the 53-kDa form of HDC was not accessible to the anti-HDC antibody in SLO-treated RBL-2H3 cells [13], which strongly suggests that the 53-kDa form of HDC is localized in the luminal compartments of the cells. Indeed, the 53-kDa form was found to be resistant to trypsin digestion in SLO-treated RBL-2H3 cells. On the contrary, the 53-kDa form was accessible to the anti-HDC antibody in SLO-treated PMN. Since HDC was not released upon SLO-treatment, HDC may be bound to the cytosolic side of granule membranes in PMN. We have no experimental evidence giving a clear explanation for this inconsistency in the localization of HDC between mast cells and PMN. Membrane orientation and targeting of HDC remains largely unknown. Using the *in vitro* translation system with rabbit reticulocyte lysates, we previously demonstrated that targeting of the 74-kDa form to the microsomal membranes occurs post-translationally [24], although the exact mechanism remains to be determined. Since the 74-kDa form of HDC lacks the amino-terminal signal sequence, which can bind to the signal recognition particle, the membrane targeting of HDC may be mediated by an unknown mechanism. Further analyses are required to clarify this mechanism.

Compared with mast cells, histamine release from PMN is characterized by high levels of spontaneous release, indicating the rapid turnover of nascent histamine in PMN. On the other hand, the observation that relatively small amounts of histamine were released upon PMA treatment indicates the existence of heterogeneous pools of granule histamine. Granule-associated localization of HDC may contribute to uptake of nascent histamine into granules, and spontaneous release of granule contents may occur in PMN infiltrated into the peritoneal cavity. The transport system for histamine across the plasma membrane and vesicular membranes remains largely unknown. There has been no experimental evidence that excludes the possibility that histamine produced in the cytosol may be directly released via a transporter in the plasma membrane. Indeed, we have recently reported that histamine is transported into the cytosol of mouse macrophages in a $Na^+$-independent manner and that cytosolic histamine is liberated from the cells after depletion of extracellular histamine, although the molecular identity of the transporter remains to be determined [25]. We surely need further experimental evidence to address this problem.

It has been demonstrated that large amounts of histamine are transiently released from mast cell granules in the anaphylactic phase of inflammatory responses,

 

whereas continuous histamine release is usually observed in the late phase, which is accompanied by an increase in histamine synthesis [9]. These distinct characteristics of histamine release may reflect the difference in the fashion of histamine synthesis and storage between mast cells and PMN; histamine is accumulated and stored in the granules of mast cells, whereas it is rapidly released from PMN.

In summary, we demonstrated in this study that HDC is induced in PMN infiltrated into the peritoneal cavity and that the dominant 53-kDa form, which is converted by a rapid post-translational processing, is localized on the cytosolic side of gelatinase-containing granules in PMN. PMN may be one of the important sources of histamine in inflammatory responses.

## 4 Materials and methods

### 4.1 Materials

An anti-HDC antibody was prepared as described [26]. This antibody was raised against the partial amino acid sequence (residues 1–210) of mouse HDC and can recognize both the precursor 74-kDa form and the mature 53-kDa form of HDC [13]. The following materials were purchased from the sources indicated: an anti-MMP-9 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); an anti-protein disulfide isomerase (PDI) antibody from StressGen (Victoria, BC); a FITC-conjugated anti-CD11b antibody and a phycoerythrin-conjugated anti-Gr-1 antibody from PharMingen (San Diego, CA); a FITC-conjugated anti-rabbit IgG antibody and a rhodamine-conjugated anti-mouse IgG antibody from Leinco Technologies, Inc. (Ballwin, MO); a rhodamine-conjugated donkey anti-goat IgG antibody and a FITC-conjugated donkey anti-rabbit IgG antibody from CHEMICON (Temecula, CA); an Alexa594-conjugated anti-rabbit IgG antibody from Molecular Probes, Inc. (Eugene, OR); polyvinylidene difluoride (PVDF) membranes from Millipore (Tokyo, Japan); a horseradish peroxidase-conjugated anti-rabbit IgG antibody from Dako (Glostrup, Denmark); ISOGEN from Nippon Gene (Tokyo, Japan); Percoll, ECL Western blot detection reagent and protein A Sepharose CL-4B from Amersham Pharmacia (Uppsala, Sweden); Biodyne A from Pall BioSupport Corporation (East Hills, NY); SLO, *N*-formyl-methionyl-leucyl-phenylalanine, and leukotriene $B_4$ from Sigma Aldrich (St. Lois, MO); [$^{35}$S]methionine (1,000 Ci/mmol) and [$\alpha$-$^{32}$P]dCTP (3,000 Ci/mmol) from Du Pont-New England Nuclear (Boston, MA). All other chemicals were commercial products of reagent grade.

### 4.2 Animals

Female BALB/c mice (7–8 weeks old) were obtained from Shimizu Experimental Animal Lab, Co. Ltd. (Kyoto, Japan). All experiments were performed according to the Guideline for Animal Experiments of Kyoto University.

### 4.3 Isolation of elicited peritoneal PMN

Female BALB/c mice were used for all experiments. Casein in saline (5%, w/v, 2 ml/cavity) was injected intraperitoneally. Peritoneal cell types were determined by microscopic observation after May-Grünwald-Giemsa staining. Cells in the peritoneal cavity were harvested 5 h after injection by lavage of the cavities with 3 ml sterile PBS. Lavage fluids were centrifuged at 200×g for 5 min at 4°C, and the pellet was resuspended in 2 ml PBS. PMN were purified by centrifugation on discontinuous Percoll gradients [27]. Briefly, 2 ml cell suspension were carefully layered on top of the discontinuous Percoll gradient prepared with 2 ml solution B (density 1.090 g/ml) and 2 ml solution A (density 1.070 g/ml). After centrifugation at 500×g for 20 min at 4°C, two distinct leukocytic layers were obtained. The lower band was collected as the PMN fraction and washed twice in PBS. Determination of the cell population by May-Grünwald-Giemsa staining indicated that more than 98% of the cells obtained were neutrophils, whereas the rest were mononuclear cells.

### 4.4 Isolation of PMN from bone marrow

Bone marrow cells were harvested from the femurs of female BALB/c mice and washed in Hanks' balanced salt solution. PMN in bone marrow were isolated according to the procedure described [28] with minor modifications. Briefly, bone marrow cells were resuspended in PBS ($5\times10^7$–$7\times10^7$ cells/ml) and carefully layered on top of the discontinuous Percoll gradient consisting of three layers [29] (3 ml each; densities 1.082, 1.075, 1.053 g/ml). After centrifugation at 500×g for 30 min at 4°C, the bottom fraction (1 ml) was collected as the PMN fraction (BM-PMN) and washed twice in PBS. Determination of the cell population was performed by May-Grünwald-Giemsa staining and by flow cytometry.

### 4.5 Culture of peritoneal and bone marrow PMN

Purified PMN or bone marrow PMN were cultured in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated fetal calf serum in a fully humidified 5%-$CO_2$ atmosphere (standard culture conditions) for the indicated times. The viability of greater than 98% of the cells was confirmed by trypan blue exclusion.

### 4.6 Flow cytometric analysis

Flow cytometric analyses were performed with a FACSCalibur (Becton Dickinson) using a FITC-conjugated anti-CD11b

 

antibody and a phycoerythrin-conjugated anti-Gr-1 antibody to determine the neutrophil populations. The population of positive cells was determined by comparison to cells stained with FITC- or phycoerythrin-conjugated isotype-matched immunoglobulin.

#### 4.7 Immunofluorescence studies

PMN were centrifuged onto round cover glasses (■=18.0 mm), which were then placed in 12-well culture plates. Immunofluorescence studies were performed as described [13]. An anti-HDC antibody (1:500), an anti-MMP-9 antibody (1:500), an anti-PDI antibody (1:500) and a FITC-conjugated anti-CD11b antibody (1:1,000) were used as primary antibodies. Alexa594-conjugated goat anti-rabbit IgG antibody (1:1,000) was used for HDC and CD11b double-staining. A rhodamine-conjugated donkey anti-goat IgG antibody (1:100) and a FITC-conjugated donkey anti-rabbit IgG antibody (1:100) were used for MMP-9 and HDC double-staining, respectively. Stained cells were observed by confocal microscopy (MRC-1024, Bio-Rad Laboratories, Hercules, CA).

#### 4.8 Northern blot analyses

Total RNA was extracted from purified PMN using ISOGEN according to the manufacturer's instructions, separated (3 μg/lane) by electrophoresis on a 1.5% agarose/formaldehyde gel and transferred onto a Biodyne A membrane in 20× SSC (1× SSC is composed of 0.15 M NaCl and 0.015 M sodium citrate) by capillary blotting. Hybridization was performed with a $^{32}$P-labeled cDNA fragment specific for murine HDC (Pvu II-digested fragment) in hybridizing solution (6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA) at 68°C overnight [30]. The filter was rinsed twice in 2× SSC containing 1% SDS at room temperature and twice in 0.2× SSC containing 0.1% SDS at 60°C. A $^{32}$P-labeled probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in rehybridization for the loading control. The filter was then analyzed using a Fujix BAS 2000 Bio-Imaging Analyzer.

#### 4.9 Immunoblot analyses

PMN were homogenized in 50 mM HEPES-NaOH pH 7.3, containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2% polyethylene glycol 300, 0.2 mM PMSF and 0.1% TritonX-100 and centrifuged at 15,000×g for 30 min at 4°C. The resultant supernatant (50 μg/lane) was subjected to SDS-PAGE (10% slab gel), and the separated proteins were transferred electrophoretically to a PVDF membrane. Immunoblot analysis was performed as described [13]. An anti-HDC antibody (1:500) or an anti-MMP-9 antibody (1:500) was used as the primary antibody, and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3,000) or a horseradish peroxidase-conjugated anti-goat IgG antibody (1:3,000) was used as the secondary antibody. The membranes were stained using an ECL kit according to the manufacturer's instructions.

#### 4.10 Histidine decarboxylase assay

PMN were rinsed with PBS followed by centrifugation, and the cell pellet was lysed ($1\times10^7$ cells/ml) with 50 mM HEPES-NaOH pH 7.3 containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2% polyethylene glycol 300, 0.2 mM PMSF and 0.1% Triton X-100 on ice for 30 min. The cells were centrifuged at 100,000×g for 1 h at 4°C, and the supernatant was used for the measurement of histidine decarboxylase activity as described [13]. The precipitate fraction exhibited no detectable enzymatic activity. The histamine formed was separated on a cation exchange column, WCX-1 (Shimadzu, Kyoto, Japan), by HPLC and then measured by the *o*-phtalaldehyde method [31].

#### 4.11 Immunoprecipitation

PMN were starved for 30 min in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and then pulse-labeled with [$^{35}$S]methionine (10 μCi/ml) for the indicated periods. In the chase experiments, 1 mM cold methionine was added to the cells after pulse labeling and incubated for the indicated periods at 37°C. Immunoprecipitation with an anti-HDC antibody was performed as described previously [13]. The dried gel was analyzed using a Fujix BAS 2000 Bio-Imaging Analyzer.

#### 4.12 Gelatin zymography

Gelatin zymography was performed according to the procedure described [32]. Briefly, SDS-PAGE was performed with 10% acrylamide slab gels containing 0.28% gelatin. After electrophoresis, gels were incubated twice in 2.5% Triton X-100 at room temperature for 30 min, and the in-gel gelatinase reaction was performed in 100 mM Tris-HCl pH 7.5 containing 10 mM $CaCl_2$ at 37°C for 12 h. Gelatin digestion was confirmed by Coomassie brilliant blue staining.

#### 4.13 Streptolysin-O treatment

PMN were incubated with SLO (12,500 U/ml; preactivated by incubation for 15 min on ice with PBS containing 10 mM dithiothreitol) in PBS at 4°C for 10 min. After this binding step, cells were rinsed twice with PBS and then incubated at 37°C for 3 min to cause permeabilization. More than 80% of lactate dehydrogenase activity was recovered in the leaked fraction under these conditions. No immunoreactive band was detected in the leaked fraction upon immunoblot analyses with the anti-PDI antibody, indicating that the ER mem-

 

brane was intact. Detergent-free buffer was used in the immunofluorescence experiments for analysis of cells after selective permeabilization of the plasma membrane.

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# 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.

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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 6] 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.[7] 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.[14–16] 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.[29] 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 $2\times10^3$ 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 $5\times10^4$ 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 $\chi^2$ 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.

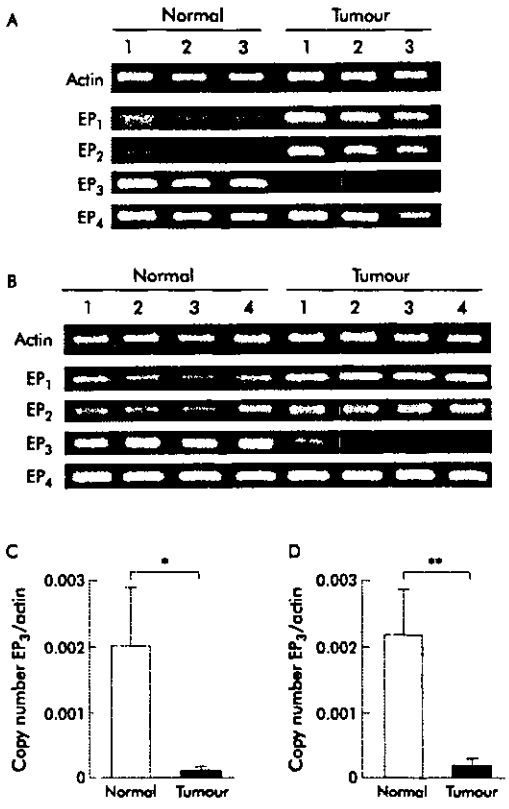


**Figure 1** Analyses of prostaglandin $E_2$ ($PGE_2$) receptors $EP_1$, $EP_2$, $EP_3$, and $EP_4$ mRNA expression. (A) Azoxymethane (AOM) treated mouse normal colon mucosa and colon carcinomas. Two pairs of samples (lanes 1, 2) and two independent samples (lane 3) were examine by reverse transcription-polymerase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

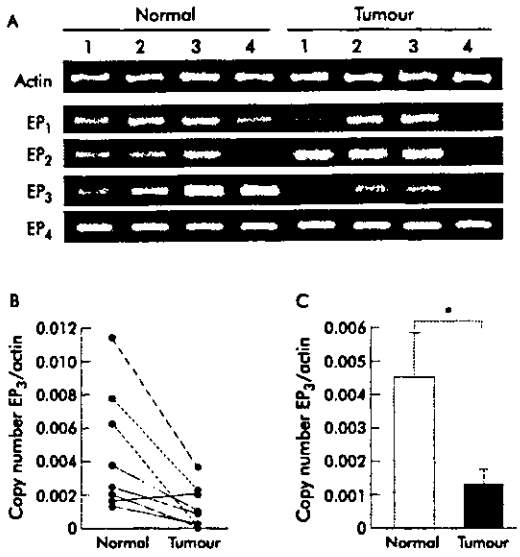


**Figure 2** Analyses of prostaglandin $E_2$ ($PGE_2$) receptors $EP_1$, $EP_2$, $EP_3$, and $EP_4$ mRNA expression in human colon tissues. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis patterns in four typical pairs of samples (lanes 1–4) are shown. (B, C) Quantitative real time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

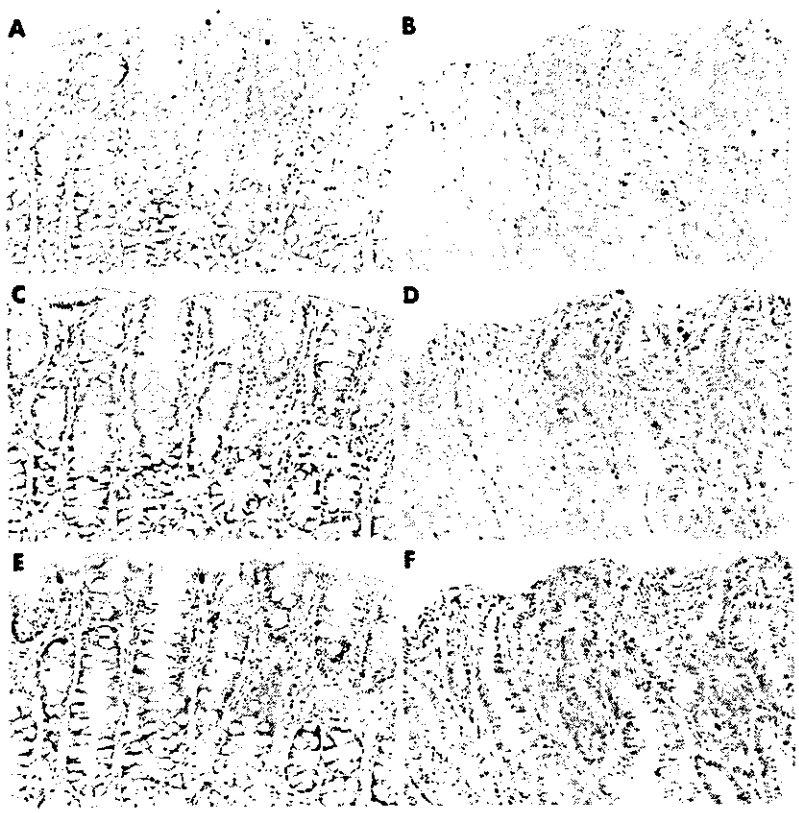


**Figure 3** Immunohistochemical staining for the rat prostaglandin $E_2$ receptor subtype $EP_3$ of normal colon mucosa (A, C, and E) and colon adenocarcinoma (B, D, and F). Non-specific staining of some red blood cells and weak background staining were observed in the negative controls stained without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

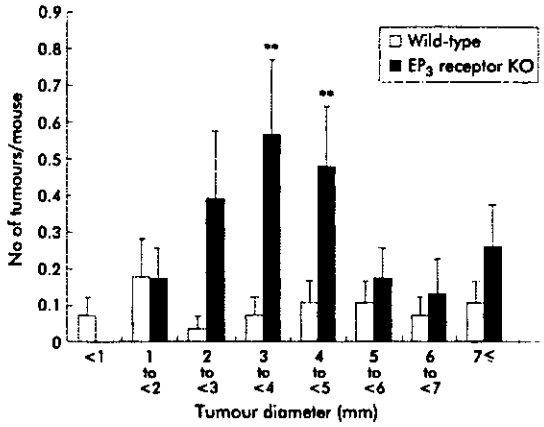


**Figure 4** Size distribution of colon tumours induced by azoxymethane in wild-type and prostaglandin $E_2$ receptor subtype $EP_3$ knockout (KO) mice. The number of tumours/mouse in each size class is expressed as mean (SEM). **Significantly different from the corresponding wild-type value ($p<0.01$).

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*$^{1716}$ mice.[19] 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.[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.

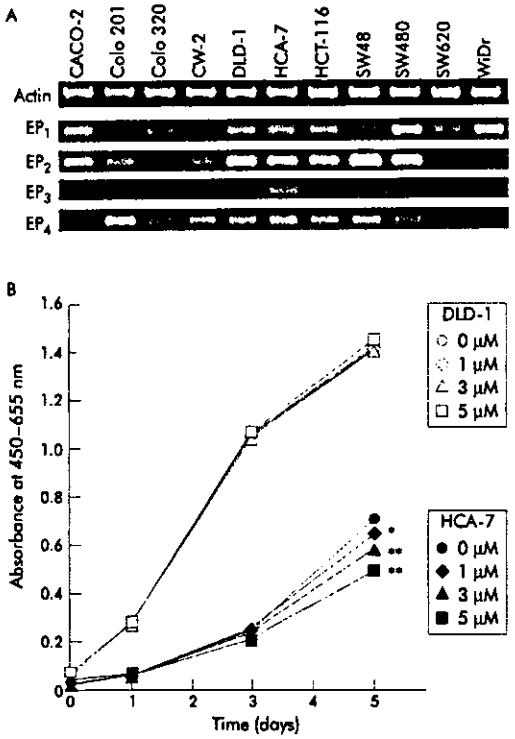


**Figure 5** Effect of ONO-AE-248 treatment on cell growth of DLD-1 and HCA-7 cells. (A) Expression of prostaglandin $E_2$ ($PGE_2$) receptors $EP_1$, $EP_2$, $EP_3$, and $EP_4$ was analysed by reverse transcription-polymerase chain reaction in 11 human colon cancer cell lines. (B) DLD-1 and HCA-7 cells were seeded onto 96 well plates at a density of $2\times10^3$ 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 ($n=6$). *$p<0.05$, **$p<0.01$.

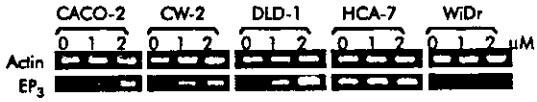


**Figure 6** 5-Aza-2′-deoxycytidine (5-aza-dC) treatment of CACO-2, CW-2, DLD-1, HCA-7, and WiDr colon cancer cell lines. Each cell line was treated with 1 and 2 μM 5-aza-dC three times. $EP_3$ receptor expression was analysed by reverse transcription-polymerase chain reaction.

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.[9, 11] 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.[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.[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.[31] 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.[17, 18] Although hypermethylation of the prostaglandin receptor gene has not been reported,[17, 18] 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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