Colonic adenoma has been suggested as the first in a series of steps leading to colorectal tumorigenesis. The COX-2 expression in macrophages of the lamina propria has been shown the therapeutic value of aspirin both in the prevention and COX-2 expression, leading to the subsequent development of colonic adenoma.

**Background and aims:** Cyclooxygenase 2 (COX-2) expression in subepithelial macrophages of colorectal adenoma has been shown. We tested the hypothesis that chemokines released from human colorectal adenoma epithelium might be involved in COX-2 expression in macrophages of the lamina propria.

**Methods:** Endoscopic samples of sporadic colorectal adenomas were tested by enzyme linked immunosorbent assay for chemokines involved in macrophage chemotaxis. COX-2 expression and prostaglandin (PG) E2 and vascular endothelial growth factor (VEGF) release, were examined in human macrophages isolated from peripheral blood.

**Results:** MCP-1 levels were markedly higher in adenoma with mild-moderate dysplasia (129.7 (19.9) pg/mg protein) and severe dysplasia (227.9 (35.4) pg/mg protein) than in normal colonic mucosa (55.8 (4.2) pg/mg protein). Other chemokine levels, macrophage inflammatory proteins (MIP)-1α and MIP-1β, and the chemokine regulated on activation of normal T cell expressed and secreted (RANTES) did not vary significantly between adenoma and normal mucosa. MCP-1 levels in both adenoma and normal colonic mucosa increased significantly three hours after tissue cultivation in vitro. MCP-1 immunoreactivity was restricted to the adenoma epithelium, with no reactivity seen in adjacent normal epithelial cells. MCP-1 stimulated COX-2 expression and PGE2 release in human macrophages. Celecoxib, a selective COX-2 inhibitor, inhibited MCP-1-induced PGE2 and VEGF release in macrophages. Addition of exogenous PGE2 reversed this inhibitory effect on VEGF release, suggesting that MCP-1 in adenoma epithelial cells might be involved in COX-2 expression and subsequent macrophage activation.

**Conclusions:** MCP-1 in colorectal adenoma epithelial cells might be involved in macrophage migration and COX-2 expression, leading to the subsequent development of colonic adenoma.
Thus we hypothesised that –CC chemokines released from epithelial cells in colonic adenoma might be involved in macrophage migration, COX-2 expression in these migrant macrophages and, thereby, in the evolution of colonic adenoma into tumorigenesis. In this study, we investigated expression of various macrophage chemotactic factors in human colorectal adenomas and examined the effect of MCP-1 on COX-2 expression, prostaglandin (PG) E₂ release, and vascular endothelial growth factor (VEGF) production in human macrophages.

**MATERIALS AND METHODS**

**Patients and tissue specimens**

Tissue samples were obtained from 156 patients (105 males, 51 females; median age 69.84 years; age range 40–88) who had undergone colonoscopy due to altered bowel habits or abdominal pain, at the Department of Internal Medicine, Nippon Medical School, Tokyo, Japan. We collected one colonic adenoma sample by endoscopic polypectomy and one adjacent normal colonic mucosa biopsy sample per patient. Adenomas and normal mucosa from patients with inflammatory bowel disease or FAP were excluded from this study. Adenoma tissue samples were fixed in 10% formalin and embedded in paraffin. Sections were prepared, stained with haematoxylin and eosin (H&E), and analysed immunohistochemically. Specimens were diagnosed and grade of dysplasia classified according to the WHO classification by expert pathologists. Specimens not diagnosed as adenomas were excluded. Finally, we used 10 adenoma samples each of these adenoma types per chemokine analysis of MCP-1, RANTES, MIP-1α, and MIP-1β. We also used 10 additional adenoma samples for short term tissue cultivation. Before examination, the protocol was fully explained to all subjects and written informed consent was obtained.

**Chemokine assays**

Adenoma tissue samples were homogenised with phosphate buffered saline (PBS) at pH 7.4 containing 2 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l peptatin A (Sigma Chemical Co, St. Louis, Missouri, USA), and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) and 1 mmol/l buffered saline (PBS) at pH 7.4 containing 2 mmol/l ethyl-pyrurate, 2 mmol/l L-glutamine, 0.1% NaHCO₃, 50 U/ml penicillin, streptomycin (Gibco-BRL) and 2% fetal calf serum (FCS; Biological Industries, Israel). Samples were centrifuged at 10 000 g for 15 minutes at 4°C and supernatants used for subsequent chemokine analysis. Briefly, 200 μl aliquots of each supernatant were added to enzyme linked immunosorbent assay (ELISA) plates coated with MCP-1 antibody (R&D Systems, Minneapolis, Minnesota, USA) and incubated at room temperature for two hours, and then for an additional 30 minutes with the secondary antibody against MCP-1. After colour development, we measured absorbance at 450 nm for MCP-1. For each adenoma polyp and normal mucosa, RANTES, MIP-1α, and MIP-1β concentrations were also quantified as per instructions in the respective ELISA kits (MIP-1α, MIP-1β, and RANTES; R&D Systems). Total protein concentration was also evaluated using the protein assay system (Bio-Rad Laboratories, Hercules, California, USA). Bovine serum albumin (Seikagaku Kogyo, Tokyo, Japan) was used as standard. Chemokine concentrations are expressed as pg per mg protein. For each grade of adenoma dysplasia, we compared adenoma chemokine levels with those of normal mucosa from the same patient.

**Adenoma tissue culture**

To verify that adenoma tissue synthesises chemokines, 10 adenoma and 10 normal colonic mucosa tissue samples were washed twice with PBS immediately after endoscopic polypectomy and then cultured in RPMI-1640 (Nikken, Kyoto, Japan) for three hours at 37°C. Residual tissue samples were fixed, stained with H&E, and analysed immunohistochemically. After three hours of cultivation, tissue samples were homogenised and supernatants used for chemokine analysis, as described above.

**Immunofluorescence analysis of human sporadic colonic adenoma**

Double immunofluorescence analysis and confocal laser scanning microscopy were used to evaluate colocalisation of COX-2 (diluted 1:5; IBL, Gunma, Japan) and MCP-1 (diluted 1:10; PePro Tech, Rocky Hill, New Jersey, USA). Sections were incubated overnight at 4°C with a mixture of the two primary antibodies. The antibody against COX-2 was allowed to react with a secondary antibody (goat antirabbit IgG; diluted 1:100; Vector Laboratories, Burlingame, California, USA) labelled with Texas red. The antibody against MCP-1 was allowed to react with a secondary antibody (horse antimouse IgG; diluted 1:100; Vector) labelled with fluorescein isothiocyanate (FITC; Sigma Chemical Co.), followed by nuclear counterstaining with 4’, 6-diamidino-2-phenylindole (Sigma Chemical Co.) for 15 minutes to facilitate identification of morphological features.

Sections were also incubated with anti-human COX-2 and anti-CDC68 (Dako, Kyoto, Japan) antibodies (both diluted 1:25 in PBS) and followed by incubation with Texas red conjugated horse antimouse IgG (IgG; 1:50; Vector) and FITC conjugated goat antirabbit IgG (COX-2; Vector) for 60 minutes at room temperature. Immunofluorescence was analysed under a laser scanning confocal fluorescence microscope (Leica TCS-4D DMIRBE, Heidelberg, Germany), equipped with argon and argon-krypton laser sources.

**Isolation of monocytes from human blood**

Peripheral blood was collected from volunteer donors. Peripheral blood mononuclear cells (PBMC) were isolated from human blood samples by Ficoll-Paque (Phamacia Biotech, Uppsala, Sweden) centrifugation. PBMCs (2×10⁶/ml) were seeded onto six well plates, incubated in RPMI-1640 supplemented with non-essential amino acids, 2 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 0.1% NaHCO₃, 50 μg/ml streptomycin (Gibco-BRL, Gaithersburg, Maryland, USA), 50 U/ml penicillin (Gibco-BRL), and 2% fetal calf serum (FCS; Trace Laboratories, Ratline, Illinois, USA) overnight, and adherent cells allowed to differentiate into macrophages. Monocytes comprised over 85% of these cells, as shown by CD68 staining, and had a viability >90%, as indicated by Trypan blue dye exclusion tests. These cells were stimulated with 10 ng/ml MCP-1 for 24 hours in the presence of FCS, and then harvested and stored at −70°C for western blot analysis. Supernatants were harvested for PGE₂ and VEGF measurements.

**PBMC preparation for immunocytochemical analysis**

Human macrophages (2×10⁶/ml) were plated onto two well Laboratory-Tek chamber slides (Nalge Nunci Intl, Naperville, Illinois, USA). After 24 hours of incubation, with or without 10 ng/ml MCP-1, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then permeabilised with methanol for 20 minutes at 4°C. Cells were then incubated with anti-CDC68 (Novocastra Laboratories, Newcastle, UK) and anti-COX-2 antibodies, and double immunofluorescence was analysed as described above.

**MCP-1 stimulation of PBMC and measurement of PGE₂ and VEGF**

PBMC were seeded onto each well of six well plates or onto 10 cm dishes at a density of 2×10⁶ cells/ml in either 1% or
10% FCS culture medium. Cells were incubated for 24 hours with either 20 ng/ml lipopolysaccharide (LPS) or 10 ng/ml MCP-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA) in the presence of 1% or 10% FCS. Thereafter, culture supernatants were used to measure PGE\textsubscript{2} and VEGF concentrations according to instructions in commercially available kits (PGE\textsubscript{2}: Assay Designs Inc., Ann Arbor, Michigan, USA; VEGF: Biosource, Camarillo, California, USA), as we have shown recently in human gastric fibroblasts.\textsuperscript{24} In experiments with COX inhibitors, either 200 nmol/l SC560 (Pharmacia, Newark, New Jersey, USA) or 10 \textmu mol/l celecoxib (Pharmacia), selective COX-1 and COX-2 inhibitors, respectively, were added one hour before MCP-1 stimulation in the presence or absence of PGE\textsubscript{2} (Sigma-Aldrich, St Louis, Missouri, USA). Previous studies have shown that SC560 at 100–300 nmol/l reduces PGE\textsubscript{2} release according to dose, without affecting COX-2 dependent PGE\textsubscript{2} production.\textsuperscript{25} Thus we used SC560 at 200 nmol/l in the present study. Celecoxib 10 \textmu mol/l has been shown to inhibit COX-2 dependent PGE\textsubscript{2} production by >90% without affecting cell viability.\textsuperscript{26}

Western blot analysis of COX-1 and COX-2 proteins in human macrophages

COX protein in human macrophages was partially purified, as previously reported by Mizuno and colleagues.\textsuperscript{27} Macrophages were homogenised in 50 mmol/l Tris HCL (pH 8.0), 0.5 mol/l sucrose containing 1.0 mmol/l PMSF, 1.0 mmol/l pepstatin A, and 2.0 mmol/l EDTA. CHAPS (3-[\(\text{N-cholamidopropyl}\)-dimethylammonio]l-propane-sulfonate) (Sigma Chemicals) was added to 1% (wt/vol), and the mixture stirred for two hours at 4°C. After 10 minutes of centrifugation at 15 000 \textit{g}, the supernatant was loaded onto an anion exchange column equilibrated with 25 mmol/l Tris HCL (pH 8.0) plus 0.4% CHAPS. The fraction eluted at 500 mmol/l NaCl was concentrated to 40% of the initial volume. Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a Hybond-P nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK) and probed with anti-COX-1 or anti-COX-2 antibody (diluted 1:50, 1:100). Bound antibodies were detected with horseradish peroxidase conjugated antirabbit IgG (diluted 1:2000) using the enhanced chemiluminescence detection system (Amersham Biosciences). Protein concentration was measured with protein assay reagent.

Statistical analysis

Data are presented as means (SEM). The paired \(t\) test was used for comparison of chemokine assays for normal colonic mucosa and adenomas in the same patients. PGE\textsubscript{2} and VEGF levels released from human macrophages were analysed by...
the non-parametric Mann-Whitney test. The software program Statview was used for data analysis. A p value <0.05 was regarded as statistically significant.

RESULTS

Chemokine levels in human sporadic colonic adenoma

We measured levels of MCP-1, RANTES, MIP-1α, and MIP-1β, all chemokines potentially involved in the migration of macrophages into the subepithelium of colonic adenoma. After pathological examination, each chemokine level was measured in 20 adenoma samples, 10 each for adenomas with mild-moderate or severe dysplasia. Each adenoma sample was matched with its corresponding normal colonic mucosa sample, taken from the same patient, and chemokine levels compared. MCP-1 levels were significantly higher in adenoma than in normal colonic mucosa (55.8 (4.2) pg/mg protein), with higher levels seen in samples of colonic adenoma with severe dysplasia (227.9 (35.4) pg/mg protein) than in those with mild-moderate dysplasia (129.7 (19.9) pg/mg protein) (fig 1A). However, there were no significant differences in chemokine levels for RANTES, MIP-1α, or MIP-1β for all groups tested (fig 1B–D).

MCP-1 synthesis in adenoma tissue cultured in vitro

To verify that elevated MCP-1 levels are derived from the adenoma itself, and not from the peripheral circulation, we cultured adenoma tissue for a brief period and compared MCP-1 levels before and after cultivation.

In this experiment, all but one tissue sample was identified as mild to moderate dysplasia by subsequent H&E analysis of residual tissue samples. Thus nine mild to moderate dysplasia samples were used for the present analysis. MCP-1 levels in both adenoma and normal colonic mucosa significantly increased during three hours of cultivation. These increases were in proportion to basal levels of each tissues type, suggesting that MCP-1 is not derived from the peripheral circulation, but actually synthesised by colonic adenoma themselves (fig 2).

Immunofluorescence analysis of MCP-1 and COX-2 in human sporadic colonic adenoma

Next we examined the immunohistological localisation of MCP-1 and COX-2 in colonic adenoma and normal mucosa. Neither COX-2 nor MCP-1 immunoreactivities were seen in normal colonic mucosa (fig 3D). In contrast, MCP-1 reactivity was detected in the cytoplasm of epithelial cells in both mild to moderate and severe dysplasia adenomas, whereas COX-2 reactivity was detected in stromal cells at the upper border of the lamina propria, just beneath the epithelium of colonic adenomas (fig 3A–C). Figure 3 shows a representative immunofluorescence analysis of adenomas with mild to moderate dysplasia. Although localisation of MCP-1 and COX-2 expression did not vary significantly between mild-moderate and severe dysplasia adenomas, only weak COX-2 expression was seen in epithelial cells of the latter (data not shown).
Immunohistological colocalisation of CD68 and COX-2 in human sporadic colonic adenoma

To examine the possibility that, in colonic adenoma, macrophages express COX-2 in the subepithelium, we performed dual immunohistochemical analysis of COX-2 and CD68 expression in macrophages. Single immunofluorescence in adenomas with mild to moderate dysplasia showed numerous interstitial CD68 positive cells (fig 4A) and a smaller number of interstitial COX-2 positive cells (fig 4B). Dual fluorescence analysis showed COX-2 protein expression largely limited to a subgroup of CD68 positive macrophages (fig 4C–D).

COX-1 and COX-2 expression in MCP-1 stimulated human macrophages

We then examined the effect of MCP-1 on COX-1 and COX-2 expression in human macrophages prepared from PBMC, as described in materials and methods. MCP-1 at 1 and 10 ng/ml stimulated macrophage COX-2 protein expression according to dose, with no effect on COX-1 expression (fig 5). COX-2 expression levels stimulated by 10 ng/ml MCP-1 for 24 hours paralleled those of COX-2 expression stimulated by 20 ng/ml LPS. Neither COX-2 nor COX-1 inhibitors had any effect on COX-2 expression levels stimulated by 10 ng/ml MCP-1.

Immunofluorescence analysis of anti-CD68 and COX-2 antibodies in human macrophages

Although our preparations of human peripheral macrophages were highly purified, contamination by other mononuclear cells present in PBMC could not be avoided. We thus examined whether COX-2 protein induced by MCP-1 stimulation was limited to macrophages, using immunofluorescence staining with anti-COX-2 and anti-CD68 antibodies. When the macrophage preparation was stimulated with 10 ng/ml MCP-1 for 24 hours, no variation was seen in
the number or shape of CD68 positive cells, although COX-2 positive staining increased to include ~90% of cultured cells. Double immunostaining clearly showed that all CD68 positive cells expressed COX-2 protein (fig 6), suggesting that cultured macrophages expressed COX-2 protein on MCP-1 stimulation.

Next we measured PGE2 release from macrophages isolated from PMBC in response to MCP-1 by enzyme immunoassay. Macrophages were stimulated with 10 ng/ml MCP-1 in the presence of either 1% or 10% FCS, where PGE2 release increased with FCS concentration even in the absence of MCP-1 (48.3 (10.4) vs 150.0 (10.0) pg/ml; \( p < 0.05 \)). However, MCP-1 at 10 ng/ml further stimulated PGE2 release even in the presence of 10% FCS (150.0 (10.0) vs 193.3 (11.5) pg/ml; \( p < 0.05 \)). Celecoxib at 10 \( \mu \)mol/l significantly inhibited PGE2 release down to levels below those induced by 1% FCS alone (fig 7). These results suggest that MCP-1 or FCS stimulated PGE2 release was dependent on COX-2.

We then measured VEGF levels in these cultured media by ELISA. MCP-1 10 ng/ml significantly stimulated VEGF release from human cultured macrophages (1119.3 (38.4) vs 1301.0 (22.7) pg/ml; \( p < 0.05 \)). Celecoxib significantly suppressed MCP-1-stimulated VEGF release (1017.6 (76.7) pg/ml; \( p < 0.05 \)), and PGE2 reversed this inhibitory effect according to dose (1559.3 (56.1) pg/ml, with PGE2 at a concentration of 100 \( \mu \)mol/l; \( p < 0.05 \)) (fig 8). Celecoxib did not affect MCP-1-stimulated COX-2 expression (fig 5) and PGE2 reversed the inhibitory effect of celecoxib on VEGF release, suggesting that the inhibitory effect of celecoxib seen in the present study is not due to its toxic effect on macrophages. Therefore, our results suggest a domino effect initiated by MCP-1 stimulation of COX-2 expression in macrophages.
and 100 experiment. standard error calculated from triplicate samples in a representative representative of three separate experiments. The error bar indicates the to dose. VEGF level stimulated with 20 ng/ml lipopolysaccharide (LPS) expression levels increased with degree of dysplasia in lamina propria of colonic adenomas in humans, and in cultured macrophages, which in turn stimulates PGE2, and culminates in VEGF release.

**DISCUSSION**

In the present study, we demonstrated for the first time that MCP-1 stimulates COX-2 expression in human macrophages isolated from peripheral blood and cultured in vitro. In addition, MCP-1 stimulated VEGF release via a COX-2/PGE2 autocrine/paracrine pathway in these isolated macrophages. Furthermore, we found that MCP-1 expression levels were higher in adenomas than in normal colonic mucosa and was found in macrophages following their migration into the subepithelial lamina propria, thereby contributing to tumour growth in the colon. However, we should note that neither MCP-1 stimulation nor celexob inhibition of VEGF release differed much from that seen at levels induced by FCS stimulation. Thus VEGF release from adenoma macrophages in vivo may also be regulated by other yet to be identified factors, in addition to the MCP-1/COX-2 pathway.

In the present study, we found that MCP-1 stimulated both COX-2 expression and VEGF release in human macrophages. MCP-1 stimulated VEGF release from macrophages was inhibited in the presence of a selective COX-2 inhibitor. Reversal of this inhibition on addition of exogenous PGE2 suggests that, in macrophages, PGE2 is a proximate signal for MCP-1 stimulated VEGF release. It is well known that VEGF is a key factor in angiogenesis, contributing to colorectal tumour growth. Therefore, our data imply that MCP-1 expressed in these adenoma epithelial cells might be, at least in part, involved in the production of VEGF by macrophages attracted into the subepithelial lamina propria thereby contributing to tumour growth in the colon. However, we should note that neither MCP-1 stimulation nor celexob inhibition of VEGF release differed much from that seen at levels induced by FCS stimulation. Thus VEGF release from adenoma macrophages in vivo may also be regulated by other yet to be identified factors, in addition to the MCP-1/COX-2 pathway.

In the present study, we found that adenoma tissue samples, when cultured for three hours, synthesised more MCP-1 protein than normal colonic mucosa samples, suggesting that MCP-1 mRNA expression and its translation into MCP-1 protein might also be higher in adenoma than in
normal colonic mucosa. Nevertheless, it has yet to be determined what regulates MCP-1 expression in adenoma epithelial cells. In mice, APC gene mutations alone induce intestinal polyposis\(^1\) where COX-2 expressing macrophages can be seen in the polyp subepithelium.\(^4\) Similarly, even mild dysplasia adenoma showed higher levels of MCP-1 expression than normal colonic mucosa in the same patient, suggesting that any gene mutation involved in induction of adenomas might also be linked to MCP-1 expression in humans. However, more work is clearly required to identify this association. MCP-1 expression is induced in epithelial cells, and to elucidate the actual interaction between adenoma epithelial MCP-1, macrophage migration, and COX-2 expression in human colorectal adenoma.

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**References**

Monocyte chemoattractant protein 1 and macrophage cyclooxygenase 2 expression in colonic adenoma

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