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REVIEW

Mast cells and inflammation-associated colorectal carcinogenesis

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Abstract Close association between chronic inflammation and cancer has been recently highlighted. Indeed, inflammatory bowel disease (IBD) has been strongly linked with an increased risk of development of colorectal cancer (CRC). Inflammatory cell-produced inflammatory mediators, such as proinflammatory cytokines and inducible enzymes, contribute to this association. In an inflammatory microenvironment, infiltrating macrophages and mast cells mediate production of these inflammatory mediators to promote growth of tumors in target tissues. In contrast to macrophages, contribution of mast cells to CRC development in inflamed colon is not well understood. This study aimed to determine the role of mast cells in inflammation-associated colorectal carcinogenesis. CRC was induced by administration of the colonic carcinogen, azoxymethane (AOM), and the tumor promoter dextran sodium sulfate (DSS) in male mast cell-deficient $WBBF_1$-*kit*$^{W/W\text{-}v}$ (W/W^v) and mast cell-normal $WBB6F_1$-+/+(WT) mice. At week 12, the W/W^v mice had markedly lower inflammation scores in the colon when compared with WT mice. The mRNA levels of colonic proinflammatory cytokines and inducible enzymes were also decreased in W/W^V mice at weeks 12 and 20, when compared with WT counterparts. Colorectal tumors, including CRC, were identified by histopathological analysis performed 20 weeks thereafter. Importantly, there were less neoplastic and preneoplastic colonic lesions in the W/W^v mice compared with the WT mice. Thus, for the first time, our study shows that mice lacking mast cells are less susceptible to inflammation-associated colorectal carcinogenesis. Our findings also suggest that mast cells and their selected cytokines could play an important role in inflammation-mediated tumorigenesis through regulation of proinflammatory cytokines and inducible inflammatory enzymes.

Keywords Inflammation and cancer · IBD · Colorectal cancer · Mast cells · $WBBF_1$-*kit*$^{W/W\text{-}v}$ mice · Cytokines

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Introduction

The association between inflammation and cancer was first perceived in the 19th century and is now accepted as an enabling characteristic of cancer [1–5]. One consistent feature of the tumor microenvironment is smoldering inflammation. Chronic and persistent inflammation increases risk of cancer development in several tissues. Approximately 25 % of cancers are associated with chronic inflammation sustained by infections (e.g., hepatitis, gastritis, and condyloma acuminatum) or inflammatory conditions of diverse origin, including inflammatory bowel disease (IBD) and prostatitis [1–5]. Inflammation and cancer are associated by two pathways: extrinsic and intrinsic. The former pathway causes smoldering inflammatory responses, and the latter is driven by inflammation-associated programs

activated by certain oncogenes and/or the inactivation of tumor suppressor genes [6].

One of the well-known inflammation-associated cancers is colorectal cancer (CRC) that develops in the setting of IBD [7, 8]. IBD, including ulcerative colitis (UC) and Crohn's disease (CD), has unknown etiology. However, the pathogenesis of IBD is currently considered to involve the interaction between genes and the environment [9]. Patients with IBD, especially UC, have a significantly increased risk of developing premalignant dysplastic lesions and malignant adenocarcinoma of the colorectum [8, 10]. Although UC-associated CRC accounts for less than 2 % of all CRCs in the general population, it is responsible for 10–15 % of deaths in UC patients [11]. The risk of CRC is thought to be increased because of several factors including degrees of inflammation and disease duration. Indeed, disease durations of 10, 20, and 30 years correspond to CRC rates of 1.6 %, 8.3 %, and 18.4 %, respectively, in IBDs such as UC and CD [12]. Even younger patients with UC have high risk of CRC [7]. Moreover, patients with UC as well as those with CRC have been increasing in Asian countries including Japan in a fashion similar to Western countries [13]. We have developed a novel animal model using the colonic carcinogen azoxymethane (AOM) and the tumor-promoter dextran sodium sulfate (DSS) that together can induce colorectal tumors in colitic mucosa [14, 15]. This model is urgently required for investigation of the pathogenesis of IBD-related colorectal carcinogenesis [8, 16, 17]. In the model, we observed numerous mast cells infiltrated within and around adenocarcinomas induced by AOM and DSS (Fig. 1) [14].

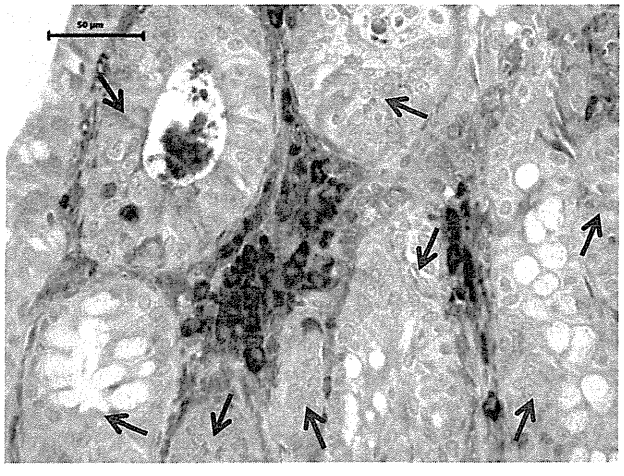

Fig. 1 Immunohistochemical staining of tryptase-positive mast cells infiltrate in the stroma of atypical glands (*arrows*) consisting of adenocarcinoma cells that developed in a male Crj: CD-1 (ICR) mouse treated with AOM and 2 % DSS [14]. Bar=50 μm

Mast cells have been reported to function in the homeostasis of wound healing and in innate and adaptive immunity. In 1891, Westphal [18] first reported a number of mast cells at the tumor periphery [19]. Since then, many animal and human studies have confirmed this observation. Thus, a biological role for mast cells in tumor progression has been suggested [20]. Indeed, enhanced infiltration of mast cells has been observed in aggressive cancers, including CRC [21]. Interestingly, mast cells are essential for intestinal polyp formation [22] and are a novel target for immunotherapy [22–24]. Additionally, mast cells have long been suspected to play a key role in IBD [25]. Mast cell number is increased in the inflamed colon of IBD patients [26] and in experimental colitis [27]. These mast cells appear to be activated as they release great quantities of inflammatory mediators and cytokines [28].

This study aimed to determine whether mast cells are involved in colitis-associated colorectal carcinogenesis initiated with AOM and promoted by DSS [14] in mast cell deficient male $WBBF_1$-*kit*$^{W/W\text{-}v}$ (W/W^v) mice [29]. The W/W^V mice that were exposed to DSS in drinking water after a single dose of AOM had significantly less colorectal neoplastic and preneoplastic lesions when compared with the WT mice. The volume of colonic adenocarcinomas, which developed in the W/W^v mice, was also significantly lower than that of WT mice. mRNA expression of several cytokines, including tumor necrosis factor (TNF)-α, in the colorectal mucosa was also lower in the W/W^V mice than WT. Taken together, our results clearly illustrate a role for mast cells as factors of both tumor promotion and progression during inflammation-mediated colorectal carcinogenesis.

Materials and methods

Animals, chemicals, and diet

Male W/W^v and WT mice (Japan SLC, Inc., Shizuoka Japan) aged 3 weeks were used in this study. They were maintained at the Institutional Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (four mice per cage) with free access to drinking water and a pellet-based diet (MFQ, Oriental Yeast Co., Ltd., Tokyo, Japan) under controlled conditions of humidity (50±10 %), light (12/12 h light/dark cycle), and temperature (23±2 °C). After arrival, the animals were quarantined for the first 7 days and then randomized by body weights into experimental and control groups. The colonic carcinogen AOM was purchased from Sigma-Aldrich (St. Louis, MO, USA). DSS with a molecular weight of 36,000–50,000 was

purchased from MP Biomedicals (Aurora, OH, USA). DSS for induction of colitis was dissolved in water to a concentration of 2 % (w/v).

Animal treatments

A total of 44 male mice of each genotype were used for the experiment. Twenty mice of each genotype were given a single intraperitoneal (i.p.) injection of AOM (10 mg/kg body weight diluted in saline). Seven days after administration of AOM, mice were exposed to drinking water containing 2 % DSS for a period of 7 days. Other experimental groups of each genotype included AOM alone (n=8), DSS alone (n=8), and untreated (n=8). At week 12, four mice of each genotype, which were treated with AOM and DSS were euthanized by CO_2 asphyxiation for analysis of mRNA expression. Colons (distalmost 1 cm) were gently removed from the underlying muscular layer with a glass microscope slide [30] for analysis of mRNA expression of the proinflammatory cytokines including nuclear factor kappaB2 (NF-κB2), TNF-α, interleukin (IL)-1β, and IL-6 as well as inducible inflammatory enzymes, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). Similarly, five mice of each genotype that received AOM and DSS and four mice each from other treatment groups (AOM alone, DSS alone, and untreated) were euthanized and colons were used for the mRNA expression analysis of the aforementioned cytokines, with the exception of NF-κB2 and inducible inflammatory enzymes at week 20 (18 weeks after the cessation of DSS exposure). At week 20, the remaining mice were also euthanized by CO_2 asphyxiation and their colons were removed for histopathological examination after hematoxylin and eosin (H&E) staining. At sacrifice, colons were removed and washed in phosphate-buffered saline. They were opened longitudinally, and feces were removed. The large intestines were then macroscopically inspected and the volume of tumors, if present, was measured. Tumor volume was calculated using two formulas: formula 1 ($a \times b^2/2$) and formula 2 ($a \times b^2 \times \pi/6$), where a and b represent the long and short diameters, respectively. For histopathological and immunohistochemical analyses, the opened colon was split longitudinally, fixed in 10 % neutral-buffered formalin, and embedded in paraffin. As did at week 12, the whole colonic mucosa without tumors was scraped with a glass microscope slide for analysis of mRNA expression of the proinflammatory cytokines (TFN-α, IL-1β, and IL-6) and inducible inflammatory enzymes (COX-2 and iNOS). Scraped samples of colonic mucosa that were collected at weeks 12 and 20 for mRNA expression analysis were placed in cryogenic vials, fast-frozen in liquid nitrogen, and stored at −80 °C until analyzed. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute, TCI-CaRP.

Histopathological and immunohistochemical analysis

Four-micrometer sections of the colon paraffin tissue blocks were stained with H&E. At week 12, inflammation in the colon of the AOM/DSS group of each genotype (n=4) was graded according to the following morphological criteria [31]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium; and Grade 4, presence of mucosal ulcer with severe inflammation (neutrophils, lymphocytes, macrophages, and plasma cell infiltration) in the mucosa, submucosa, muscularis propria, and/or subserosa. The scoring was made on the distal 1/2 colon with or without proliferative lesions and expressed as mean average score per mouse. At week 20, large bowel lesions were evaluated for the presence of mucosal ulcers, preneoplasms (dysplastic crypts), adenoma, and adenocarcinoma [14, 32]. For assessment of the presence or absence of mast cells, histology sections from the colons of both mouse genotypes were immunohistochemically stained with 500-fold diluted anti-tryptase antibody (AA1 clone, Dako Japan, Kyoto, Japan), as described previously [33].

Quantitative RT-PCR analysis

Total RNA was extracted from scraped colonic mucosa homogenates using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. cDNA was then synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analysis of individual cDNAs was performed with the ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; *Nf-kappaB2*, Mm00479807_m1; *Tnf-α*, Mm00443258_m1; *Il-1β*, Mm00434228_m1; *Il-6*, Mm00446190_m1; *inos*, Mm00440485_m1; and *β-actin*, Mm00607939_s1). PCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The expression level of each gene was normalized to the β-actin expression level using the standard curve method.

Statistical analysis

All measurements were statistically analyzed using the unpaired t test (two-tailed), Tukey multiple comparison post

test or Fisher's extract probability test. Differences were considered to be statistically significant at $p<0.05$.

Results

General observations

Several mice of each genotype that received DSS had bloody stools at week 2, but no clinical symptoms were observed thereafter. The mean percent weight loss for each genotype did not significantly differ during the DSS exposure (2 % for the W/W^V mice and 6 % for the WT mice). The daily intake (milliliter per day) of DSS-water was also insignificant between the two genotypes (13.98±0.82 for the W/W^V mice and 13.52±0.62 for the WT mice). At week 12, the mouse colons of both genotypes that were used for analysis of mRNA expression had a few and small colonic tumors located in the rectal region of the colon. These small tumors were histopathologically determined to be tubular adenoma or tubular adenocarcinoma. No significant changes in the mean body weight, liver weight, or colon length were noted among the groups of both genotypes at weeks 12 and 20 (data not shown).

Colonic inflammation score at week 12 and incidence and multiplicity of large bowel preneoplasms and neoplasms at week 20

At week 12, the colonic inflammation score of the W/W^V that received AOM and DSS (1.25±0.50, $p<0.01$) was significantly lower than the WT mice (3.00±0.81) given AOM and DSS, as illustrated in Fig. 2. At the end of the study (week 20), large bowel dysplastic crypts, tubular adenoma, and tubular adenocarcinomas developed in both mouse genotypes that received AOM and DSS, but not in either genotype when treated with AOM alone, DSS alone, or untreated group. The incidence and multiplicity of mucosal ulcer (Fig. 3a), dysplastic crypts (Fig. 3b), and colonic tumors, both adenoma (Fig. 3c) and adenocarcinoma (Fig. 3d), were determined. For the mucosal ulcers, the values were lower in the W/W^V mice (42 % incidence with a multiplicity of 0.83±1.19) than in the WT mice (80 % incidence with a multiplicity of 1.60±1.35), although this difference was not statistically significant. For the dysplastic crypts, incidence and multiplicity were higher in the W/W^V mice (83 % incidence with a multiplicity of 2.67±1.87) but were comparable to those of WT mice (80 % incidence with a multiplicity of 2.30±2.63). Similarly, as quantified in Fig. 4a and b, the incidence (58 %) and multiplicity (1.17 ±1.19) values of tubular adenoma in the W/W^V mice were close to those of the WT mice (60 % incidence with a multiplicity of 0.80±0.92). For the incidences of colonic adenocarcinoma (Fig. 4a and b), the value (83 %) of the W/W^V mice was lower than that (100 %) of the WT mice but was not statistically significant. However, the multiplicity of adenocarcinoma was significantly lower in the W/W^V mice (1.42±0.90, $p<0.005$) when compared with that of WT (4.80±3.32). Similarly, the multiplicity of total tumors (adenoma+adenocarcinoma, Fig. 4a and b) of the W/W^V mice (2.58±1.31, $p<0.02$) was significantly lower than that of WT (5.60±3.89). Figure 5 shows H&E-stained sections made from the colons of both genotypes. The mean volumes of tumors developed in the W/W^V mice (Fig. 5a, tumor number=28, Formula 1: 0.80±0.89 mm^3, $p<0.0004$ and Formula 2: 1.05±0.65 mm^3, $p<0.0009$), which were calculated by two formulas, were significantly lower than those of the WT mice (Fig. 5b, tumor number=50, Formula 1: 3.45±3.71 mm^3 and Formula 2: 3.62±3.88 mm^3). Thus, these data suggest that mast cell deficiency impairs adenocarcinoma multiplicity and tumor growth in a colitis-associated CRC mouse model.

Distribution of tryptase-positive mast cells in the colonic mucosa

To examine the presence and localization of mast cells in the colons of W/W^V- and WT-treated mice, tryptase immunohistochemistry was performed. Numerous tryptase-positive mast cells were present in the lesion-free colonic mucosa,

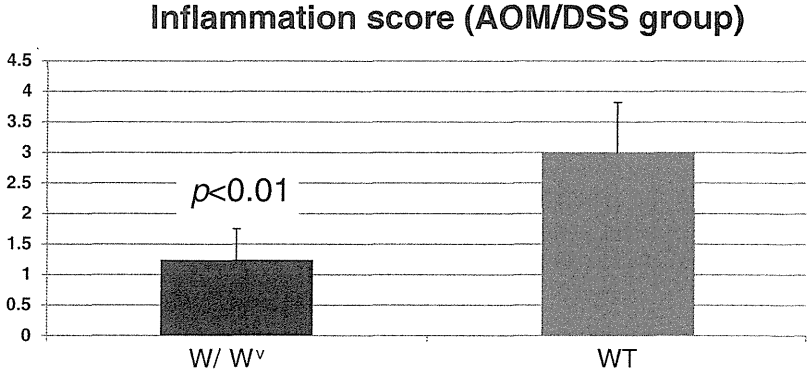

Fig. 2 Inflammation score of the colons of the W/W^V (n=4) and WT mice (n=4) that received AOM and DSS. The value of the W/W^V mice was significantly lower than the WT mice ($p<0.01$)

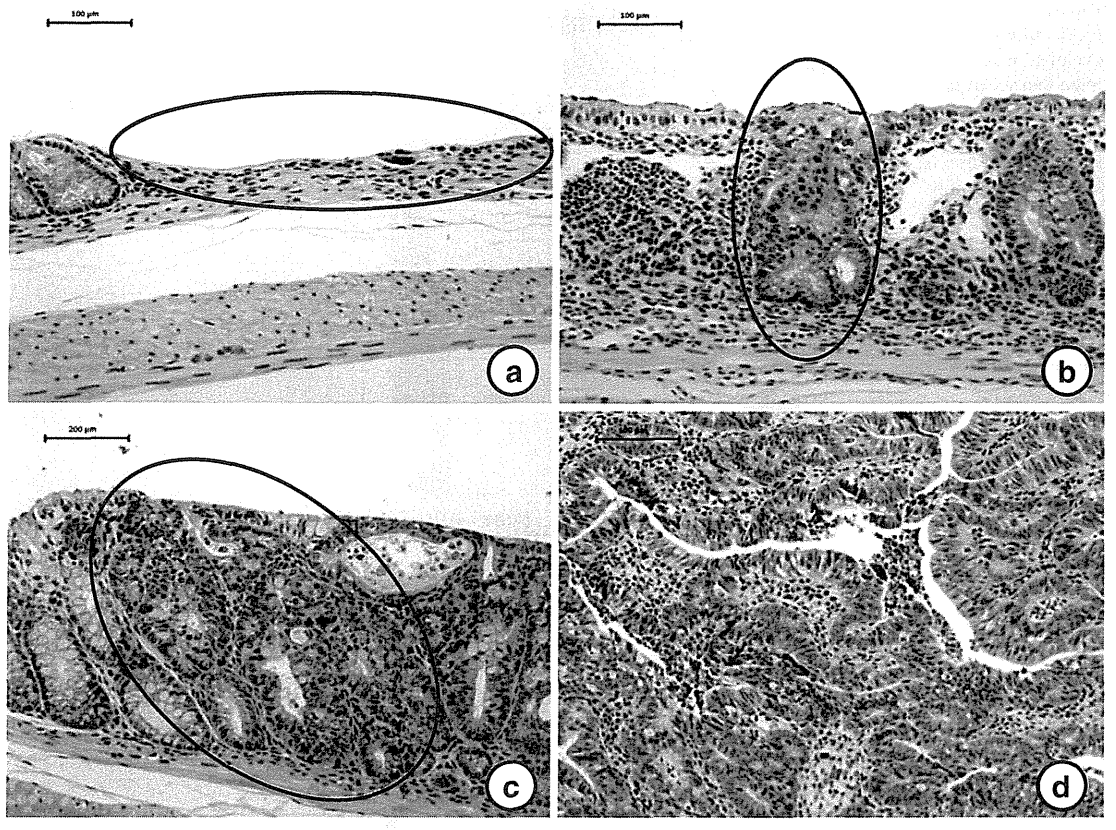

Fig. 3 Histopathology of colonic lesions developed in both the WT and W/W^V mice. Histological sections were stained with H&E and key feature are indicated: mucosal ulcer (**a**, *circled*), dysplastic crypts (**b**, *circled*), tubular adenoma (**c**, *circled*), and tubular adenocarcinoma (**d**). Bars=100 μm

adenoma, and adenocarcinoma of the WT mice that received AOM and DSS (Fig. 6a, c, and e), while none or few were present in the W/W^V mice treated with AOM and DSS (Fig. 6b, d, and f). Thus, these data reiterate that mast cells are recruited during colitis and CRC in mice. In addition, these data validate the mast cell-deficient W/W^V mouse model.

mRNA expression of proinflammatory cytokines (NF-κB2, TNF-α, IL-1β, and IL-6) and inducible inflammatory enzymes (COX-2 and iNOS)

Next, the mRNA expression profiles of key cytokines and inducible inflammatory enzymes were analyzed using colons from WT and W/W^V mice after induction of CRC. The mRNA expression of the cytokines NF-κB2, TNFα, IL-1β, and IL-6, as well as the inducible inflammatory enzymes, COX-2 and iNOS, were examined in the colonic mucosa of W/W^V and WT mice at weeks 12 and 20, respectively. At week 12, expression of all the cytokines and inducible inflammatory enzymes examined were significantly lower in the colonic mucosa of the W/W^V mice that received AOM and DSS than that of similarly treated WT mice. At week 20, the expression levels of the proinflammatory cytokines (TNFα, IL-1β, IL-6, and COX-2) and inducible inflammatory enzymes (COX-2 and iNOS) in the colonic mucosa of the W/W^V mice that received AOM and/or DSS remained lower than that of the WT mice, but the differences between the two genotypes were not statistically significant. These findings suggest that mast cell deficiency alters the colonic inflammatory profile in our colitis-related CRC mouse model.

Discussion

In this study, we investigated AOM/DSS-induced colorectal carcinogenesis in W/W^V and WT mice to determine a possible role for mast cells in inflammation-associated colorectal carcinogenesis. Our findings clearly indicated that W/W^V mice that lack mast cells were less susceptible to AOM/DSS-induced colorectal carcinogenesis when compared with similarly treated WT mice. Also, two genotypes have different degrees of inflammation in the colon (Fig. 2) and their differences in tumor volume and multiplicity might be due to this factor. The results, thus, suggest the importance of inflammatory stimuli in the AOM and DSS models.

In the current study, tryptase-positive mast cells were not identified in the colonic mucosa of the W/W^V mice by immunohistochemical staining, suggesting that this

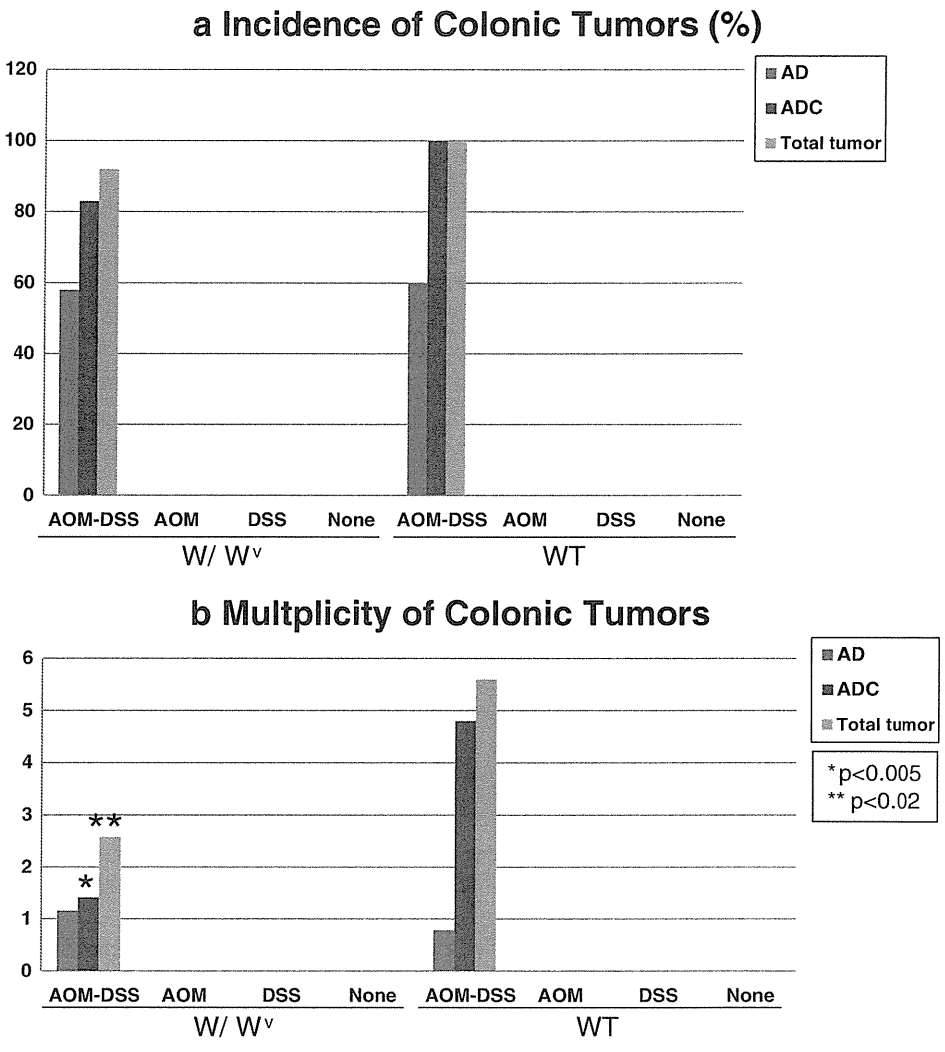

Fig. 4 Incidence (**a**) and multiplicity (**b**) of colonic tumors developed in the W/W^{v} and WT mice that received AOM and DSS. "Total tumor" refers to the combination of adenoma and adenocarcinoma. A Fisher's extract probability test and a Tukey multiple comparison post test were performed to examine the difference in tumor incidence and multiplicity between WT and W/W^{V} mice, respectively (adenocarcinoma, $p<0.005$ and total tumors, $p<0.02$)

genotype is void of mature, morphologically identifiable mast cells of both connective tissue and mucosal types [29, 34]. Mast cells both elaborate and express receptors for several cytokines including NF-κB2, TNF-α, IL-1β, and IL-6, thus implicating them in a role as intermediates between innate and adaptive immune responses [7, 35–38]. Factors involved in the pathogenesis of IBD include altered innate and adaptive immune systems, oxidative stress, microbes, several cytokines, and induced inflammatory enzymes [7]. Despite intense interest, the pathogenesis of IBD remains poorly understood. However, the imbalance between pro- and antiinflammatory cytokines is thought to play a pivotal role in modulating colonic inflammation [39]. In UC and CD, which are major types of IBD, certain proinflammatory mediators, such as TNFα,IL-1β, and IL-6, are released in inflamed mucosa of the colorectum. TNF-α and IL-1 share a multitude of proinflammatory properties and play a critical role in amplifying mucosal inflammation in IBD [40]. Both cytokines are primarily secreted by inflammatory cells upon activation and induce intestinal macrophages, neutrophils, fibroblasts, and smooth muscle cells to elaborate prostaglandins (PGs) and other inflammatory mediators. The effects of TNF-α on inflamed colon include disruption of the epithelial barrier, induction of apoptosis of the crypt cells, and secretion of chemokines from the intestinal crypt cells [40]. NF-κB is a critical transcription factor involved in a broad range of biological processes, including immune regulation, chronic inflammation, and tumorigenesis of a variety of cancers [41]. During the inflammatory process, IL-1β and IL-6 are produced and exert various effects through activation of the NF-κB signaling pathway. IL-1β is also considered the gate keeper of inflammation [42]. Together, these cytokines and activated mast cells are involved in the development, recurrence, and exacerbation of human IBD [28, 43]. Moreover, mast cell-derived tryptase plays an important role in experimental colitis [44, 45] and human IBD [25, 46, 47]. In addition to these proinflammatory cytokines, nitric oxide (NO) and

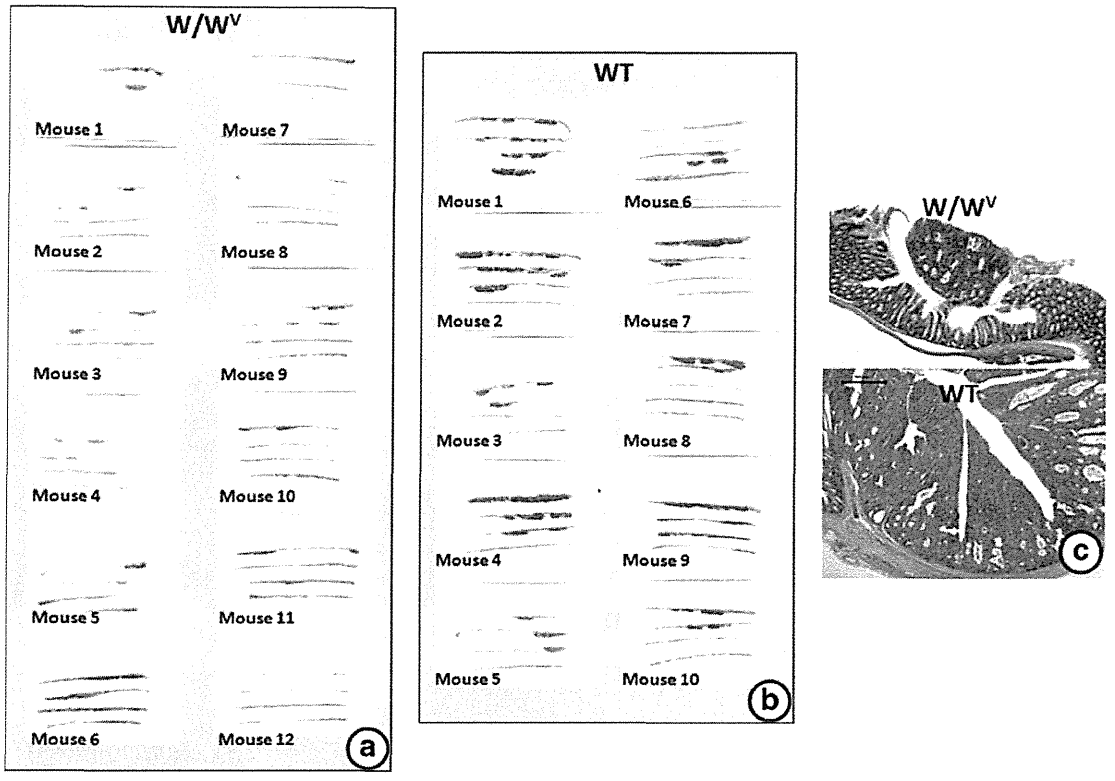

Fig. 5 Colonic tumors on the histologic sections from the W/W^V (**a**) and the WT mice (**b**). **c** Representative tumor size difference between a WT and W/W^V mouse in the histologic sections. A total of 31 and 56 colonic tumors developed in the W/W^V and WT mice, respectively. The number and size of tumors developed in the W/W^V (n=28) and WT mice (n=50) were quantified. A Tukey multiple comparison post test and an unpaired t test (two-tailed) were performed to examine differences in the tumor number and size between the W/W^V and WT mice, respectively

PGE_2 are also released at sites of inflammation during chronic inflammation [41]. Nitrosative stress caused by iNOS-derived NO is strongly associated with IBD progression and contributes to the pathogenesis of human IBD, experimental colitis [48], and colitis-associated colon tumorigenesis [49]. COX-2 is another factor involved in IBD [50] and colitis-related colon carcinogenesis [49] by acting through the synthesis of PGE_2.

In carcinogenesis, a role for mast cells in cancer development and progression has been suggested [20, 51, 52]. It has been reported that mast cells act to promote the "angiogenic switch" during tumor growth [53]. Mast cell infiltration within or around tumors, such as CRC [54], is associated with poor prognosis, although the association depends on the type of cancer [51]. In the W/W^V mice, tumor growth of subcutaneously injected B16–BL6 melanoma cells has been shown to decrease at early stages but was recovered after reconstitution of the mast cell population [55]. Mast cells are reported to be causatives in colonic adenoma formation, the early step of colorectal carcinogenesis, in humans [22]. In this study, the expression of TNF-α, a major product of mast cells [56] that acts at the apex of proinflammatory pathways in IBD [57] and promotes CRC [58], was decreased in the colon of mast cell-negative W/W^V mice compared with WT mice after induction and promotion of CRC. TNF-α was most significantly decreased when expression was examined 12 weeks after initiation with AOM. At this same time point, expression of NF-κB2 in the colonic mucosa of W/W^V mice was lower than that of the WT mice. NF-κB is considered to play a pivotal role in immune and inflammatory responses through the regulation of genes encoding proinflammatory cytokines and inducible enzymes, such as COX-2 and iNOS. Thus, our findings suggest that NF-κB, as well as cytokines and inducible enzymes, are rationale targets for either prevention or treatment of colitis and colitis-related colon carcinogenesis [59].

In colitis-associated CRC, DNA injury is induced by production of free radicals and the iNOS system in the colonic mucosa with persistent inflammation, followed by *p53* mutation and development of a precancerous lesion, dysplasia. Dysplasia progression to invasive CRC is mediated by COX-2, iNOS, and several cytokines produced by infiltrating inflammatory cells and a result of the accumulation of genetic abnormalities, such as loss of the *DCC* gene [8]. In this study, we observed the expression of COX-2 and

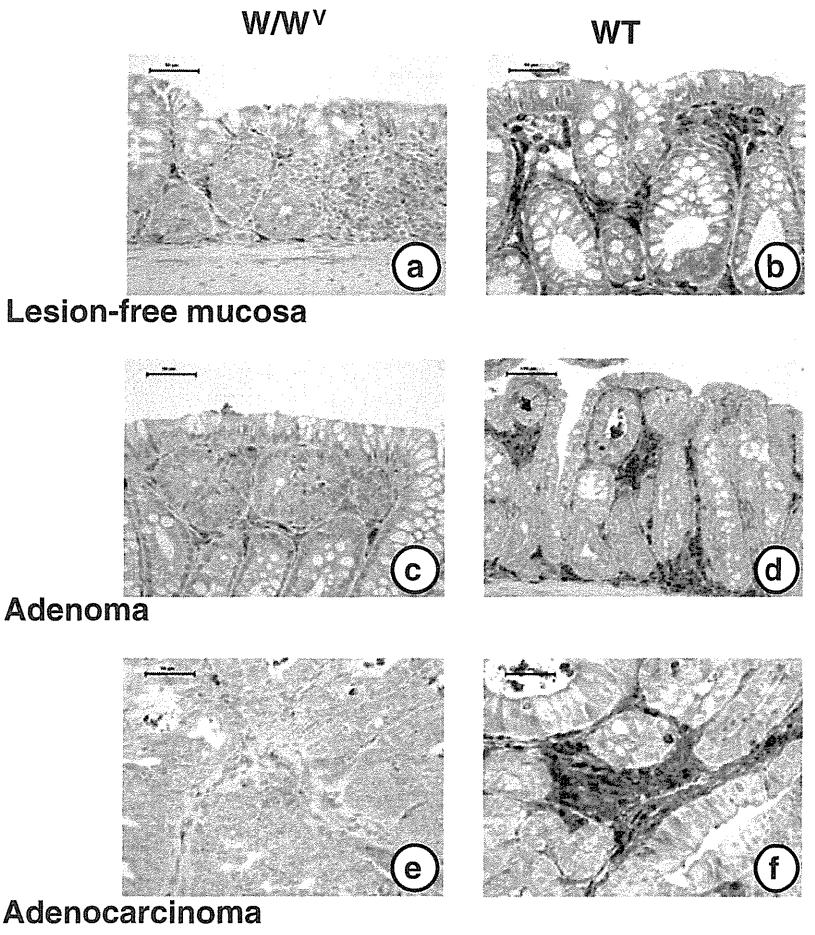

Fig. 6 Staining for tryptase-positive mast cells in lesion-free colonic mucosa (**a** and **b**), adenoma (**c** and **d**), and adenocarcinoma (**e** and **f**) from the W/W^V and WT mice. **a** and **b** are from mice that did not receive AOM and/or DSS. **c**–**f** are from mice that received AOM and DSS. Bars=50 μm

iNOS in the colonic mucosa of W/W^V mice at two sacrifice points. Both inducible inflammatory enzymes are targets for therapy and prevention of IBD and CRC [60, 61], since they are elevated in the colorectal mucosa of IBD patients [62, 63] and involved in gastrointestinal carcinogenesis [63, 64]. Experimentally, iNOS-produced bone marrow-derived cells play critical roles in mediating the inflammatory response during colitis [65]. iNOS-derived NO is, thus, a potential target for developing therapeutics for inflammatory diseases, including IBD [66].

There exist several limitations in this study. We did not investigate whether gut microflora is similar between the two genotypes of mice. Since gut microflora influences AOM/DSS-induced colorectal carcinogenesis [67], we should confirm that gut microflora is similar between the two strains in future studies with use of siblings from mating, etc. The experimental cancer model used in these studies does not fully approximate the human disease. IBD-associated CRC in humans is thought to progress from dysplasia without a sporadic adenoma intermediary step [8]. CRC in the AOM/DSS model may develop from dysplastic crypts or adenoma [15]. Despite this, a high frequency of chromosomal instability was detected in CRC induced by the AOM and DSS treatment [68], while CRC produced by AOM alone did not show chromosomal instability [69]. Since human colitis-associated CRC [70, 71] as well as UC [72] has high frequency of chromosomal instability, the AOM/DSS model could provide a valuable tool to investigate molecular pathogenesis of colitis-associated CRC development.

We also determined that the W/W^V mice displayed less colon inflammation than the WT control mice (Fig. 2). It remains to be determined whether the mast cell dependent on carcinogenesis in this model can be explained by this difference in inflammation versus other intrinsic pathways related to tumor growth and development. However, our findings provide further evidence in support of the concept that collaboration between aberrant epithelium and cellular components of the tumor microenvironment is important for carcinogenesis. In particular, the development and maintenance of an inflammatory microenvironment greatly contribute to colorectal carcinogenesis in inflamed colon. Our data provides experimental evidence supporting mast cells as important modulators of inflammation-associated colorectal carcinogenesis. Therefore, we would like to propose that mast cells deserve more focused consideration as a therapeutic target in IBD and IBD-related CRC.

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Conflict of interest None.

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Cancer Medicine

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ORIGINAL RESEARCH

Preventive effects of low-dose aspirin on colorectal adenoma growth in patients with familial adenomatous polyposis: double-blind, randomized clinical trial

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Abstract

There are several reports of clinical trials of aspirin in sporadic colon cancer. However, only one double-blind trial of aspirin in patients with familial adenomatous polyposis (FAP) has been reported to date. This double-blind, randomized, placebo-controlled clinical trial was therefore performed to evaluate the influence of low-dose aspirin enteric-coated tablets (100 mg/day for 6–10 months) in 34 subjects with FAP (17 each in the aspirin and placebo groups). The increase in mean diameter of colorectal polyps tended to be greater in the placebo group compared with the aspirin group, which showed a response ratio, that is, aspirin response rate (number of subjects with reduced polyps/total)/placebo response rate (number of subjects with reduced polyps/total), of 2.33 (95% confidence interval: 0.72–7.55). Subgroup analysis revealed that the number of subjects with a mean baseline polyp diameter of $\leq$2 mm, and the diameter and number of polyps after intervention showed a significant reduction in the aspirin group. Adverse effects of aspirin, such as anastomotic ulcer, aphtha in the large intestine, and progression of anemia, occurred in three subjects. Moreover, none of the subjects developed colorectal cancer. The results thus indicated a potential for aspirin to reduce colorectal adenoma development in patients with FAP, but careful follow-up is needed to avoid or rapidly counter severe adverse effects.

Introduction

Familial adenomatous polyposis (FAP) is a very rare autosomal dominant inherited disorder, characterized by the occurrence of many adenomas in the large intestine. It has been reported that the polyps in the colorectum progress to adenocarcinoma in half of the patient population by the age of 40 years [1].

Hitherto, complete surgical removal of the colorectum was the only preventive method against colorectal cancer

development in FAP patients. This intervention, however, has caused frequent diarrhea, which led to a significant decrease in patients' quality of life. Therefore, research on chemoprevention has been conducted in the hope that it may be able to delay surgery or, in patients with a smaller number of polyps, help avoid surgery by combining it with endoscopic resection [2]. Among candidate substances, sulindac, a nonsteroidal anti-inflammatory drug (NSAID), has been studied frequently in a clinical setting [3–5]. Giardiello et al. reported that sulindac reduced the number and size of colorectal adenomas in a double-blind randomized trial [3]. Although many articles indicate that sulindac may safely inhibit colorectal adenoma, there is just one article that reported the possibility that long-term administration of sulindac may cause adverse effects such as gastric ulcer and anemia [4]. Although hopes have focused on cyclooxygenase-2 (COX-2)-selective inhibitors, including celecoxib and rofecoxib, causing little damage to the gastric mucosa [6, 7], recent studies have revealed that they may evoke cardiotoxicity [8, 9].

In the recent past, a great deal of experience has been gained with long-term use of aspirin tablets for antiplatelet therapy for cardiovascular diseases around the world. This treatment is relatively well tolerated and clinical trials have demonstrated protective effects of aspirin against sporadic colorectal adenomas as well as colorectal cancers [10–13]. However, to our knowledge, only one clinical trial of aspirin in patients with FAP has been reported to date [14]. Here, we report efficacy and safety of low-dose aspirin enteric-coated tablets for suppression of intestinal polyp development in FAP patients, with special attention to control of adverse effects.

Materials and Methods

Trial methodology

In this double-blind, randomized, placebo-controlled trial using low-dose aspirin enteric-coated tablets, the subjects received either 100 mg/day aspirin or placebo for 6–10 months. The duration of 6–10 months was selected because the periodic colonoscopy for FAP patients was 6–10 months. Random assignment was performed in each case by an investigator using a computer-aided system on the Medical Research Support Web site (Kyoto, Japan). This web site was available only to the trial participants. We used a minimization algorithm, one of the dynamic allocation methods for clinical trials, to achieve a balance between treatment groups with respect to four stratification variables: institution, sex, age (<30 or ≥30 years), and history of colorectal surgery. Enrollment of the subjects started in June 2007 and the trial was completed in October 2009. Osaka Central Hospital and the University of Occupational and Environmental Health Hospital participated in this trial, for which an Ethics Monitoring Committee was established. A system to ensure continuous follow-up of adverse effects was also installed. Each of the two trial sites obtained approval from its own ethics committee for the conduct of the trial.

Trial population

The trial population was patients with FAP, defined as the presence of ≥100 adenomas in the large intestine, or a germline mutation in the adenomatous polyposis coli (*APC*) gene. All the subjects participating in the trial had an intact rectum or a residual rectum at least 2 cm in length, were aged ≥16 and ≤70 years, and were Japanese. As FAP patients with preserved colonic mucosa had received colonoscopy regularly and already had polyps 5 mm or larger removed endoscopically, there was no polyp of 5 mm or larger at the time of recruitment.

The following were excluded from the trial: (1) patients with active cancer at the time of entry, (2) those currently taking an antithrombotic or anticoagulant, (3) individuals with a history of stroke or gastric/duodenal ulcers (except for those with scars healed as confirmed after successful eradiation of *Helicobacter pylori*), (4) those with inflammatory enteritis, hemorrhagic diverticulitis, or bleeding tendency, (5) those with a platelet count of ≤100,000/mm^3, those with abnormal prothrombin time, (6) people with known allergy to aspirin, (7) those who were pregnant or planned to become pregnant during the trial period, and (8) those taking an NSAID for pain relief more than three times weekly. Subjects who had previously been treated with sulindac were allowed to participate in the trial with the limitation that the drug had been discontinued more than 6 months prior to the start of the trial.

Consent interviews were individually performed and informed consent regarding participation in the trial was obtained from each patient.

Investigational drug

Low-dose aspirin enteric-coated tablets (100 mg per tablet) and placebo counterparts provided by Bayer in Germany were imported to Japan. This trial was financed by research funding by the Ministry of Health, Labour and Welfare. We signed an agreement to assure that we had no conflict of interest for Bayer. The investigational drugs were placed in blister packages (calendar sheets of 31 tablets), both sides of which were aluminum laminated.

Study questionnaire

At the trial entry, height, body weight, medical history, and use of NSAIDs were investigated for each patient through a questionnaire. In addition, data regarding everyday meals were collected using a self-administered food-frequency questionnaire developed by the Department of Health and Nutrition, Nagoya-bunri University, Aichi, Japan [15].

To ensure accurate characterization of adverse effects and evaluation of tolerance, the subjects were asked to keep a patient diary, such as drug compliance and medical conditions, documenting their condition during treatment, with a blister sheet sent to the data center every month.

Trial end points

Colonoscopy to measure the number, diameter and height of polyps was performed at two time points, before the start and at the end of the trials, as follows. Only one visual field per subject, in which at least four polyps could be detected, was tattooed with ink before the start of the trials. For subjects who had more than one area of multiple polyps, the field from which the best frontal view of the polyps could be obtained was selected. We took several photos using a clamp with a 2-mm scale placed near the tattooed area. Then we measured the diameter and height of the polyps using the scale shown in the photos. Prior to randomization code breaking, changes of polyp were assessed by the Data Evaluation Committee in a blinded manner, comparing endoscopic photographs taken before and after aspirin treatment. The primary end point was set as the presence or absence of an increase in mean diameter of all colorectal polyps observed compared with that before taking aspirin. The secondary end points included change in polyp height, mean diameter and number of polyps, and adverse effects.

Immunohistochemical staining

Colorectal polyps sampled during colonoscopy were fixed, embedded, and sectioned for immunohistochemical examination with the avidin–biotin complex immunoperoxidase technique, using a polyclonal goat anti-COX-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a polyclonal goat anti-β-catenin antibody (Santa Cruz Biotechnology) at 100× dilution. As the secondary antibody, biotinylated anti-goat IgG (Vector Laboratories Inc., Burlingame, CA) was employed at 200× dilution. Staining was performed using avidin–biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3′-diaminobenzidine, and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As negative controls, consecutive sections were immunostained without exposure to the primary antibody.

Statistical analysis

The target sample size was initially set at 100 (50 per group), with the following rationale. To obtain 20% polyp regression in the placebo group and 50% in the aspirin group, 39 subjects were needed for each group to ensure a power of 80% with a two-sided 5% significance level. Considering the possibility of trial dropouts, the target number was increased.

Baseline characteristics were tested by the chi-square test or the Student's *t*-test. "Response rate" was defined as the percentage of subjects with reduced polyp size and number after drug administration among all the subjects of the same group compared with preadministration. Response ratio = aspirin response rate (number of subjects with reduced polyps/total)/placebo response rate (number of subjects with reduced polyps/total). Change in polyp quantitative data was tested by calculating the response ratios and 95% confidence intervals. Adverse event rates of both arms were compared using the chi-squared test. Fisher's exact probability was applied, if needed due to sparse cells in a table. These analyses were also performed in several subgroups. All statistical analyses were intention-to-treat based and performed using PC-SAS (Version 9.1; SAS Inc., Cary, NC) with *P*-values less than 0.05 considered statistically significant.

Results

Recruitment of subjects

A total of only 51 patients could be recruited, of whom 35 (69%) provided informed consent, and took aspirin or placebo tablets. When 10 subjects underwent the end-of-trial colonoscopy, an anastomotic ulcer and severe anemia with decrease to below 3 mg/dL of hemoglobin were observed in one subject receiving aspirin. Consequently, the co-researchers and the Ethics Monitoring Committee decided to cancel further recruitment. Of note, the randomization results were not disclosed until the end of the trial. To conduct strict monitoring during follow-up, we frequently checked the subjects for abdominal pain or symptoms of anemia. We were ready to discontinue trial drug administration and perform blood tests, colonoscopy, and upper esophagogastroduodenoscopy earlier than planned if these conditions developed in the subjects. Fortunately, no subject after the first subject randomized presented with abdominal pain or symptoms of anemia during the trial,

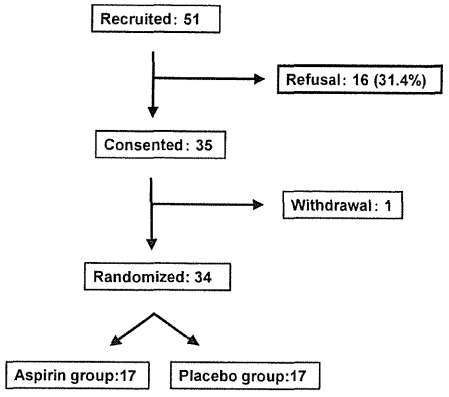

Figure 1. Flowchart for subject recruitment.

and all subjects except for one subject were able to complete trial drug administration as scheduled. The subjects who had already agreed to participate in the trial continued to take the aspirin or placebo with close monitoring of their symptoms (Fig. 1). In addition, one of the subjects who consented to participate in the trial was not included because of involvement in a severe traffic accident before assignment. Thus, 17 subjects each were allocated to the aspirin and placebo groups and completed the trial.

Characteristics of subjects

The characteristics of the subjects of aspirin and placebo groups are shown in Table 1. There were no statistically significant differences between the two groups regarding sex, age, surgical history, drinking status, smoking status, and *APC* gene mutation.

Table 1. Characteristics of subjects in the aspirin and placebo groups.

	Aspirin group (n = 17)	Placebo group (n = 17)
Age (years), mean ± SD	39.7 ± 12.8	36.7 ± 13.9
Body mass index,[1] mean ± SD	22.4 ± 3.4	21.7 ± 2.1
Treatment period[2] (days), mean ± SD	250.2 ± 58.0	255.2 ± 57.6
Compliance (%), mean ± SD	83.3 ± 23.8	88.4 ± 10.6
Number (%) of subjects		
Male	8 (47)	9 (53)
Female	9 (53)	8 (47)
Current smoker	4 (24)	4 (24)
Alcohol drinker[3]	7 (41)	5 (29)
Undergone colectomy with IRA	4 (24)	3 (18)
Mean tumor diameter <2 mm	14 (82)	11 (65)
With *APC* gene mutation[4]	14 (82)	12 (71)

SD, standard deviation; IRA, ileorectal anastomosis.
[1]Body mass index = weight (kg)/height (m) squared.
[2]Data obtained at end of trial.
[3]Alcohol drinker: drinks more than once a week.
[4]*APC* gene mutation was detected by protein truncation test assay.

Effect of aspirin on polyps in FAP patients

Subjects in the aspirin group tended to demonstrate greater reduction in the diameter of their colorectal polyps, the primary end point, than the subjects in the placebo group, with a response ratio of 2.33 (95% confidence interval: 0.72–7.55). However, the difference obtained in a response ratio was not statistically significant (Table 2).

Subgroup analysis revealed that number of subjects with a mean baseline polyp diameter of ≤2 mm had a significant reduction in the aspirin group (Table 2). Five of 14 subjects had a significant reduction in the number of polyps in the aspirin group, while no patient had a change in the placebo group (Table 2). Although we should view this small-size study with caution, the diameter of polyps before intervention was almost the same in the aspirin group (1.66 ± 0.61 mm [mean ± SD]) and

Table 2. Number of subjects with reduced polyps in aspirin and placebo groups.

	Aspirin group (number of subjects with reduced polyps/total)	Placebo group (number of subjects with reduced polyps/total	Response ratio	95% Confidence interval
All subjects	7/17	3/17	2.33	0.72–7.55
Subjects with mean polyp diameter at baseline				
≤2 mm	5/14	0/11	P = 0.046[1]	
>2 mm	2/3	3/6	1.33	0.43–4.13
Polyp height	10/17	5/17	2.00	0.87–4.62

[1]Fisher's exact probability test.

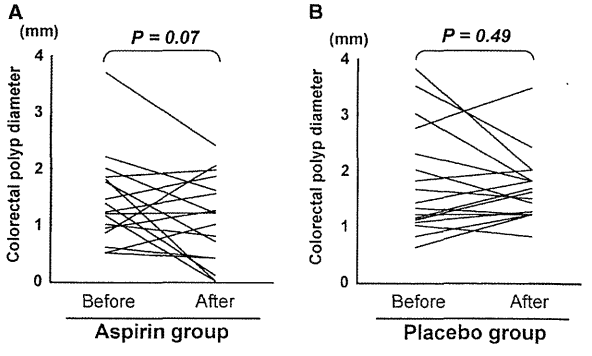

Figure 2. Endoscopic evaluation of polyp size. (A) Changes of mean colorectal polyp diameter in aspirin group. (B) Changes of mean colorectal polyp diameter in placebo group.

placebo group (1.78 ± 0.96 mm). After intervention, the diameter of polyps significantly decreased in the aspirin group (1.09 ± 0.75 mm [$P < 0.05$]) compared with that in the placebo group (1.41 ± 0.78 mm) (Fig. 2). Moreover, the number of polyps was smaller in the aspirin group than in the placebo group after intervention. The number of polyps before intervention was almost the same in the aspirin group (2.82 ± 1.54 [mean ± SD]) and placebo group (2.53 ± 1.38). After intervention, the number of polyps significantly decreased in the aspirin group (2.18 ± 1.69 [$P < 0.05$]) compared with that in the placebo group (2.53 ± 1.38). The polyp height tended to exhibit a greater decrease in the aspirin group with a response ratio of 2.0 (Table 2). Thus, the number of subjects with a mean baseline polyp diameter of $\leq$2 mm, and the diameter and number of polyps after intervention were shown to be the items that achieved significant difference in the subgroup analysis. Moreover, none of the subjects developed colorectal cancer.

Adverse events of aspirin on FAP patients

There were no serious adverse effects in the placebo group. Of 17 subjects assigned to the aspirin group, three experienced severe adverse effects (18%) ($P = 0.23$, vs. placebo group). These effects included anastomotic ulcer, aphtha in the large intestine, and progression of anemia (3 mg/dL reduction of Hg). All of these subjects were nonsmoking women aged <40 years with high β-catenin staining of their polyps on immunohistochemical examination. A giant anastomotic ulcer was detected at the suture site of the ileal porch in one of the subjects (Fig. S1); therefore, the drug was discontinued after colonoscopy. The ulcer healed as confirmed by endoscopy at 6 months after discontinuation. Aphtha in the large intestine in the second subject was confirmed to have disappeared at the next colonoscopy. Anemia in the third subject improved after cessation of exposure to aspirin. These two subjects had no history of surgery.

Discussion

In this double-blind, randomized trial, low-dose aspirin enteric-coated tablet administration tended to reduce the size of colorectal polyps in subjects with FAP, as compared with the placebo group. Furthermore, subgroup analysis indicated that the number of subjects with a mean baseline polyp diameter of $\leq$2 mm had a significant reduction in the aspirin group. Moreover, the diameter and number of polyps significantly decreased, and polyp height tended to decrease. However, we could not draw a definite conclusion from the subgroup analysis as the sample size was too small.

Loss of the *APC* gene and/or dysfunction of APC is known to influence degradation of β-catenin, increasing the levels of β-catenin, and leading to activation of COX-2 through β-catenin/Tcf-4 transcription complexes [12]. It is well established that prostaglandins produced by COX-1 and COX-2 play important roles in colorectal cancer development, and aspirin blocks the activity of both of these enzymes [16]. Moreover, 80% of sporadic colorectal cancers feature *APC* gene somatic mutations, which may appear very early during colorectal carcinogenesis [17]. Thus, the present results indicated that aspirin might act at a relatively early stage of colorectal tumor development through inhibition of prostanoid synthesis.

Recently, Burn et al. reported the results of their CAPP1 study, an international, multicenter, randomized, placebo-controlled trial of aspirin (600 mg/day) and/or resistant starch (30 g/day) for 1–12 years in FAP patients (10–21 years of age) [14]. According to their report, there were no adverse effects due to aspirin, but no reduction of polyps was observed either.

In this clinical trial, we could not achieve the targeted number of subjects because adverse effects occurred during the course of the investigation. All of our subjects who experienced adverse effects were in the aspirin group, and improvement was observed after discontinuation of exposure, suggesting a causative relationship. Adverse effects, such as anastomotic ulcer, aphtha in the large intestine, or progression of anemia was observed in 3 of 17 subjects. We do not have any evidence to explain this high incidence of adverse effects. It is considered that unexpected genetic backgrounds, such as polymorphisms, mutations, and epigenetic changes, might have affected aspirin metabolism. Further examination is required to clarify the reason. Adverse effects due to aspirin in healthy individuals have been reported to occur more frequently in elderly people [18], so that there might be differences between healthy individuals and subjects with FAP in this respect. In the previous clinical trial of sulindac reported by ourselves [4], the similar very high incidence of adverse effects in FAP subjects supported this conclusion. In our previous trial on sulindac, one patient developed a gastric perforation requiring emergency surgery. In this trial, again a serious adverse effect, a giant anastomotic ulcer, was observed. We thought the effect was too severe to continue further recruitment, although gastrointestinal mucosal injury was one of the predictable adverse effects. In future clinical trials of low-dose aspirin enteric-coated tablets in this high-risk group, dose reduction in young women or in those with *APC* gene alteration, or concomitant use of a proton pump inhibitor might be considered.

We would like to emphasize that the low-dose aspirin enteric-coated tablet used in the trial is identical to that

widely used for antiplatelet treatment across the world, including Japan. Obvious advantages are the great deal of experience with its long-term use and good safety profile. It was reported that the incidence of adverse effects (including mild ones) was 2.67% [19] according to post-marketing surveillance in Germany conducted by Bayer, or 6.54% according to the survey on Kawasaki disease in Japan by the Ministry of Health, Labour and Welfare [20].

Interestingly, our three subjects with marked symptoms of adverse effects had a relatively large tumor size reduction in common, possibly indicating shared sensitivity for both beneficial and adverse effects. It is necessary to develop a method to identify patients who would benefit from aspirin with a low risk of adverse effects, and to determine the optimal dose. A recently developed technology, as evidenced by a genome-wide association study [21], may help in this respect.

There were several limitations of this trial. First, the sample size was small and second, the evaluation was limited to the tattooed area, without covering the entire colon. However, the reasons we chose these conditions were that it was difficult to objectively evaluate the changes in polyps in the entire colon as it might increase the burden on patients, and that other studies conducted to date also used a similar evaluation method to ours.

In conclusion, the potential for aspirin to reduce colorectal adenoma growth or development in patients with FAP is indicated. However, careful follow-up is needed to avoid and promptly treat adverse effects, with efforts made to identify and verify characteristics of sensitive patients.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Giant anastomotic ulcer detected by colonoscopy.

Original Research

A Pilot, Randomized, Placebo-Controlled, Double-Blind Phase 0/Biomarker Study on Effect of Artepillin C–Rich Extract of Brazilian Propolis in Frequent Colorectal Adenoma Polyp Patients

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Key words: artepillin C, colon cancer, myocardial creatine phosphokinase, cyclin D1, laxative, 8-OHdG

Objective: Brazilian propolis, a folk medicine, is used worldwide as an alternative medicine to prevent colon cancer. The objective of the study was to test in a small pilot biomarker study in a high-risk group the safety and efficacy of propolis for colon cancer prevention, which has not been evaluated in humans.

Methods: Subjects with adenoma polyps recently removed from the colon were randomly assigned to a propolis group of 15 and a placebo group of 16. In a double-blind study, the propolis group received capsules containing 165 μmol artepillin C and 150 μmol other polyphenols per day for 3 months. Prior to and at the end of the experiments, their blood was analyzed using biochemical tests, and specimens from the normal-appearing sigmoid colon mucosa were biopsied endoscopically to examine the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and mRNA expressions of proliferating cell nuclear antigen, cyclin D1, and Bax.

Results: Propolis extract significantly increased the mRNA level of cyclin D1 in the sigmoid colon mucosa, and the other biomarkers remained unchanged. Blood biochemical tests showed significantly higher activity of creatine phosphokinase (CPK), 143 ± 52 units/ml in the propolis group and 104 ± 38 units/ml in the placebo group ($p = 0.026$), at the end of the study. The increase in CPK activity in the propolis group was due to the increase of the myocardial band form of CPK. On the other hand, laxative treatment prior to endoscopic biopsy significantly increased 8-OHdG levels.

Conclusions: The results from our pilot study did not provide evidence that Brazilian propolis was effective in preventing changes occurring during early stages of colon cancer. In contrast, propolis may have detrimental side effects on muscle tissue, including myocardial cells.

INTRODUCTION

Colon cancer is a lifestyle-related disease closely associated with the daily diet [1–3] and is potentially preventable by modifying the diet [4,5]. In Japan, colon cancer is the fourth leading cause of cancer death [6]. Recently, the age-standardized rate of mortality from colon cancer has decreased slightly [7], but the age-standardized incidence remains the fourth highest in Japan [8] and the highest in the world [9]. An urgent issue is to find biomarkers to enable us to understand intermediate end points that alter early before the reliable end point of adenomatous polyp formation, since it often takes several years for a normal crypt cell to undergo molecular changes to develop and become clinically detected as adenocarcinoma [10].

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Many reports have discussed preventative factors in vegetables, fruits, and teas, and potential protective agents have been identified in phenolics [5,11,12]. Propolis has been used as a folk medicine by various populations and usually includes unique phenolics because it is a resinous material gathered by honeybees from the buds and bark of indigenous plants. In Brazil, honeybees collect propolis from a tree, *Baccharis dracunculifolia*, which particularly contains artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) [13]. Most phenolics undergo conjugations with glucuronide and/or sulfate of the functional groups and become inactive during the intestinal absorption process [14]. Among the various phenolics, artepillin C has 2 prenyls attached to the hydroxyl group, which is the target of conjugation enzymes. We therefore assumed that these 2 prenyls make it difficult to access the conjugation enzyme [15]. Indeed, most dietary artepillin C was incorporated into the blood circulation unchanged in form without conjugation in animals [16]. In colon cancer model mice induced by azoxymethane, extracts of Brazilian propolis significantly suppressed the formation of precancerous lesions and aberrant crypt foci, and the active compound was artepillin C [17,18].

The preventative mechanisms of artepillin C were the induction of arrest at the G_0/G_1 phase in the cell cycle of tumor cells by suppressing the phosphorylation of retinoblastoma protein and the release of transcription factor E2F by stimulating the expression of phosphorylation-inhibitory protein $p21^{WAF1/Cip1}$ [19] and by suppressing the oxidative formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in DNA [15]. Thus, the preventative mechanisms of colon cancer have been clarified for artepillin C, and safety data have been provided for Brazilian propolis by its history of safe use as folk medicine. The question is whether it is actually effective in humans.

In the present study, we aimed to determine the effects of Brazilian propolis extracts containing abundant artepillin C on the risk of colon tumor formation. High-risk colon cancer patients who had recently had colon adenomatous polyps removed were enrolled in a randomized, double-blind, placebo-controlled intervention study, and were examined for the effects on normal-appearing sigmoid colon mucosa using potential intermediate end point biomarkers for colon cancer as follows: levels of 8-OHdG, a biomarker for oxidative DNA damage [20]; mRNA levels of proliferating cell nuclear antigen (PCNA) [21] and cyclin D1 [22], biomarkers for cell proliferation; and mRNA levels of Bax, a biomarker for apoptotic cell death [23,24].

MATERIALS AND METHODS

Test Material

Brazilian propolis was imported and refined by alcohol extraction at Yamada Apiculture Center, Okayama, Japan. The extract of 1 g dry matter was composed of artepillin C (550 μmol), ferulic acid (36 μmol), *p*-coumaric acid (130 μmol), caffeic acids (130 μmol), kaempferol (30 μmol), kaempferide (89 μmol), 6-methoxy kaempferol (110 μmol), naringenin (51 μmol), isosakuranetin (9.3 μmol), chrysin (18 μmol), and other substances, mainly wax, when determined by the method of Sakakibara et al. [25]. Capsules for the dose consisted of 100 mg of the extract dry matter with mixed starch (40 mg), reduction starch syrup (57 mg), and SiO_2 (3 mg). Placebo capsules were prepared using starch (80 mg) and reduction starch syrup (120 mg). Both capsules were colored brown to make the brown color of propolis extract invisible and to shade them from light, and capsules were stored in a refrigerator before administration. Artepillin C content had decreased by 13% at the end of the study compared to the content at the start of experiments.

Subjects

Participants were recruited between August 2006 and April 2007 among patients who were undergoing colonoscopy at Osaka Central Hospital, Japan. Men and women were eligible if they were between the ages of 40 and 75 years and had had colon tumors removed endoscopically without colon resection. Exclusions included certain medical conditions, propolis intake, allergy to propolis, and the use of supplements such as *Agaricus*, active hexose correlated compound, shark cartilage, and so on. Initially, participants were excluded if they were receiving treatment for hyperlipidemia, hypertension, diabetes, and hyperuricemia, but this exclusion was lifted after enrollment. The study protocol was approved by the Ethics Committee of Osaka Central Hospital, and written informed consent was obtained from all study participants.

Study Protocol

This pilot study was a 3-month, randomized, placebo-controlled, double-blind intervention study evaluating the effects of artepillin C–rich extract of Brazilian propolis on colon cancer formation, using biomarkers for oxidative DNA damage, cell proliferation, and apoptotic cell death in normal-appearing mucosa of the sigmoid colon as potential intermediate end points for colon cancer. Since this study is, to our knowledge, the first human trial to assess the effect of artepillin C–rich extract of Brazilian propolis on colon cancer risk, we designed a pilot study with a minimum sample from a statistical viewpoint. Eligible subjects were randomly assigned to a propolis or placebo group. The groups were blinded to both subjects and investigators until the study was completed. Only in cases of the utmost necessity, such as serious adverse events, did the coordinator of the study allow the investigator to know the groups to which the subjects were assigned. Osaka Central Hospital participated in this study, for which an Ethics Monitoring Committee was established.

Intervention with Propolis. Subjects received 3 capsules per day, 1 capsule after every meal, for 3 months. One subject in the propolis group stopped for 1 of the 12 weeks because of persistent diarrhea not related to this study. The propolis group received 165 μmol artepillin C and 150 μmol other phenolics per day. Adherence to the intervention was >98% evaluated both by counting the empty PTP sheets of capsules that were returned to the investigators and by a self-recorded diary of doses.

At baseline, subjects provided information on height, weight, supplement use, alcohol intake, history of smoking and illness, physical activity, and dietary intake using a baseline questionnaire. Daily intake of selected nutrients was calculated based on food frequency questionnaire tests as described previously [26]. Side effects of propolis were examined by blood analyses described below and by questionnaire for common sensations (sleep state, fatigue, appetite, diarrhea, constipation, concentration, state of mind, thinking, shoulder stiffness, and sensation of warmth), both at baseline and at the end of the intervention.

Biopsy. Before intervention at the baseline in the afternoon and after intervention at the end of the study in the morning, 4 biopsy specimens of around 5 mg were obtained endoscopically from normal-appearing mucosa 20 cm from the anal verge. Biopsy specimens were immediately immersed in a RNA stabilization reagent (RNAlater; Qiagen GmbH, Germantown, MD) and placed on ice in the dark until just before analyses. A laxative was used prior to the biopsy at the baseline, but it was not used at the final biopsy because the use of a laxative had seemed to increase the evaluation value of 1 of the biomarkers when the baseline data were analyzed.

Blood Analyses. At baseline and final examinations, blood was taken from all volunteers, and the number of erythrocytes, leukocytes and platelets, the ratios of thrombocytes, neutrophilic cells, acidophilic cells, basophilic leukocytes, lymphocytes and monocytes, hemoglobin level, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and erythrocyte sedimentation rate were determined. Biochemical blood tests analyzed the total protein, albumin, transaminases, alkaline phosphatase, lactate dehydrogenase, γ-glutamyltransferase, urea nitrogen, creatinine, urea, creatine phosphokinase (CPK) [27], total cholesterol, triglyceride, and sensitive C-reactive protein.

Measurement of 8-OHdG Levels in Colon Mucosa

Levels of 8-OHdG in biopsy specimens were determined using the method of Kasai [20], with slight modification to avoid artificial formation in the dark and at low temperature. Biopsy specimens were rinsed in 0.85% NaCl, homogenized, and lysed in 10 mM Tris-HCl buffer (pH 7.5) containing 320 mM saccharose, and 5 mM $MgCl_2$, and then centrifuged at 10,000*g* for 20 seconds at 4°C. Precipitated nuclei were rinsed twice with the same buffer containing 1% (v/v) Triton X-100, followed by centrifugation and lysation in 190 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM disodium ethylenediaminetetraacetate (EDTA) and 1% (w/v) sodium dodecyl sulfate. The lysate was treated with 0.26 mg/ml ribonuclease A (Sigma, St. Louis, MO) at 37°C for 30 minutes and with 0.25 mg/ml proteinase K (Sigma) for 90 minutes, and then mixed with 50 μl of 5 M NaCl. DNA was precipitated in 50% (v/v) isopropanol for 15 minutes at −80°C and centrifuged at 17,000*g* at 4°C for 15 minutes. After washing with ice-cold 70% (v/v) ethanol and drying, DNA was dissolved in 110 μl of 1 mM EDTA, and a 10 μl aliquot was submitted to spectrophotometric examination of the purity and concentration of DNA.

This 100 μl aliquot was heated at 95°C for 5 minutes, immediately cooled on ice, treated with 5 units of nuclease P1 (Wako Pure Chemical, Osaka, Japan) in 225 μl of 33 mM sodium acetate buffer at 37°C for 30 minutes, and then with 3 units of alkaline phosphatase (Sigma) in 80 μl of 0.4 M Tris-HCl (pH 7.5) at 37°C for 60 minutes. After centrifugation at 17,000*g* at 4°C for 15 minutes, the supernatant was filtered through a 0.2 μm membrane filter (Millipore, Bedford, MA), and a 50 μl aliquot underwent high-performance liquid chromatography (HPLC) analyses of 8-OHdG and 2′-deoxyguanosine (dG), determined with a coulometric electrochemical detector (Coulochem III; ESA, Chelmsford, MA) and a UV detector (L7420; Hitachi, Tokyo, Japan) set at 290 nm, respectively. The Coulochem detector was equipped with a guard cell (Model 5020, 400 mV) and analytical cell (Model 5011, detector I, 150 mV; detector II, 350 mV). The HPLC conditions were as follows: pump, LC10AD (Shimadzu, Kyoto, Japan); reverse-phase column, Capcell pack C18 UG 120 (Shiseido, Tokyo, Japan); mobile phase, a mixed solvent of 6% methanol and 94% potassium phosphate (20 mM, pH 4.5) containing 0.1 mM EDTA; and flow rate, 1.0 ml/min. The amount of 8-OHdG was expressed as the number of 8-OHdG in 10^5 of dG.

Measurement of mRNA Levels of Cyclin D1, PCNA, and Bax in Colon Mucosa

Biopsy specimens in RNA stabilization reagent RNAlater were submitted to semiquantitative analysis of the mRNA expression of cyclin D1, PCNA, and Bax with the multiplex reverse transcription-polymerase chain reaction (RT-PCR) technique using porphobilinogen deaminase (PBGD) as the internal standard [28,29]. PCR was performed in a total volume of 25 μl reaction mixture containing 1 μl cDNA template; 1× Perkin Elmer PCR buffer; 1.5 mM $MgCl_2$; 0.8 mM deoxynucleotide triphosphate; 20 pmol of each primer for cyclin D1, PCNA, or Bax; 4 pmol each of PBGD; and 1 unit of TaqDNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Belleville, NJ). The sequences of these PCR primers and PBGD were described previously [28–30].