RESEARCH COMMUNICATION

Inhibition of Azoxymethane-induced Colon Carcinogenesis in Rats due to JTE-522, a Selective Cyclooxygenase-2 Inhibitor

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Abstract

Prostaglandin E_2, which is produced by cyclooxygenase (COX) during arachidonic acid metabolism, is considered to be related to colon carcinogenesis and selective COX-2 inhibitors may be effective for chemoprevention without the adverse side effects of non-selective, nonsteroid anti-inflammatory drugs. Therefore, the influence of JTE-522 (4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzensulfonamide), a selective COX-2 inhibitor, was examined in azoxymethane(AOM)-induced rat colon carcinogenesis. A total of 40 male F344 rats were randomly divided into two groups. Group 1 received diet containing 0.015% JTE-522 and group 2 the normal diet without supplement as a control group; one week later, all rats were administered axozymethane (AOM) s.c. at a dose of 15 mg/kg body weight once a week for 3 successive weeks. At the termination of the experiment (30 weeks after the start), the multiplicity of colon cancer in group 1 was significantly less than that of group 2. The proliferating cell nuclear antigen (PCNA) indices for non-neoplastic cells of the colon mucosa in group 1 were also lower. These data thus suggest that JTE-522 has chemopreventive potential against colon carcinogenesis with decrease of mucosal cell proliferation in rats.

Keywords: JTE-522 - selective cyclooxygenase-2 inhibitor - colon carcinogenesis - cancer chemoprevention

Introduction

Colorectal cancer is the third most common neoplasm in the world (Kohno et al., 2002). In Japan, its incidence has been increasing, and it is now the third leading cause of cancer death. For instance, just in 1995, there were 30201 men and 22266 women diagnosed as having colorectal cancer (The Research Group for Population-based Cancer Registration in Japan, 2000). Since the problem is so large, the possibility of chemopreventive approaches is of obvious interest. Based on epidemiological studies, non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin are now well established to reduce colorectal cancer risk (Yoshimi et al., 1997). In patients with familiar adenomatous polyposis, the administration of another agent, sulindac, decreased the size and number of colon adenomas (Kakizoe, 2003). Moreover, in animal models, several NSAIDs, like aspirin, piroxicam, sulindac and indomethacin, have demonstrated chemopreventive effects against chemically induced colon carcinogenesis (Kakizoe, 2003).

Since NSAIDs are cyclooxygenase (COX) inhibitors and prostaglandins, especially PGE_2, are modulators of cellular proliferation, one possible mechanism of action of the NSAIDs is through their impact on PG synthesis from arachidonic acid. In fact, PGE_2 levels in cancerous tissues have been found to be elevated as compared with corresponding normal-appearing tissues in humans and rats (Reddy et al., 2000). Two isozymes of COX, one of the rate-limiting enzymes in PG synthesis (Jones et al., 1993), have been identified in the rat, constitute COX-1 and inducible COX-2 (Sano et al., 1995). While COX-1 exists in most tissues and is involved in the physiological production of PG2 under normal homeostasis, COX-2 is induced by mitogens, cytokines and growth factors, and is responsible for production of PGs in inflammation (Eberhart et al., 1994). In human colon cancers, COX-2 expression has been shown to be increased rather than COX-1 expression. Overexpression of COX-2 mRNA in rat colon carcinogenesis has also been observed (Oshima et al., 1996). Traditional NSAIDs which inhibit both COX-1 and COX-2 have side

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effects, causing gastrointestinal ulceration and renal toxicity through the inhibition of constitutive COX-1. Therefore, attention has become concentrated on specific inhibitors of COX-2 (Reddy et al., 2000).

JTE-522 (4-(4-cyclohexyl-2-methylozazol-5-yl)-2-fluorobenzensulfonamide) (Fig.1), a novel selective COX-2 inhibitor, has been demonstrated to be a highly selective and irreversible inhibitor of both rat and human COX-2 (Mitsushita et al., 1997). Three different animal models of intestinal cancer cell growth have been used to test the antitumorigenic properties of non-selective and COX-2 selective NSAIDs: mice with adenomatous polyposis coli (APC) mutations, a nude mouse xenograft model, and chemical carcinogen-induced colon carcinogenesis in rodents (Oshima and Taketo, 2002). JTE-522 has been shown to significantly reduce the number and growth of polyps in Apc^{4474} knockout mice (Sasai et al, 2000; Sunayama et al., 1999). However, the APC mouse model is somewhat limited as a model of colon carcinogenesis in that most of the tumors are small bowel adenomas rather than colorectal cancers. In the nude mouse xenograft model, JTE-522 suppressed tumor growth of human head and neck squamous carcinoma cells (Nishimura et al., 1999) and human lung cancer cells (15). JTE-522 also inhibited liver and lung metastases of colon cancer that showed COX-2 expression but lacked any effects on samples lacking COX-2 expression (Tomozawa et al., 1999; Nagatsuka et al., 2002). Recently, another study found that JTE-522 could inhibit 1,2-dimethylhydrazine (DMH)-induced colonic aberrant crypt foci (ACF), which is a preneoplastic marker of colon carcinogenesis, in a F344 rat model (Wei et al., 2003). The present study was performed to determine whether the agent might inhibit colon carcinogenesis when given concomitantly with AOM, a metabolite of DMH, in rats. To cast light on possible mechanisms of action, cell proliferative activity in colonic epithelium was also evaluated using the proliferating cell nuclear antigen (PCNA) index assessed by immunohistochemistry.
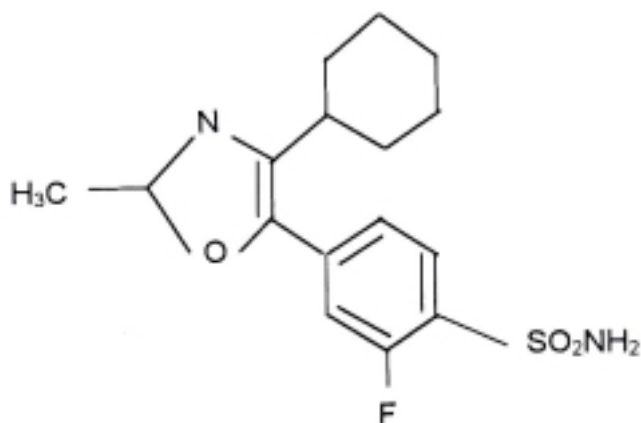

Figure 1. Chemical Structure of JTE-522

Materials and Methods

Chemicals

JTE-522 (purity, 100%) was kindly provided by Japan Tobacco Inc., Tokyo. AOM (CAS: 25843-45-2) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals and Husbandry

Male Fischer 344/DuCrj rats were purchased at 5 weeks of age from Charles River Japan Inc. (Atsugi), housed four to a plastic cage, and fed basal diet modified NIH powder diet (Oriental Yeast Co., Ltd, Tokyo) and water *ad libitum*. The animals were kept in an air-conditional barrier-system at a temperature of $22 \pm 2^\circ C$ and a humidity of 55 ± 5 % with a 12:12 light: dark cycle. They were used in this study after a 1-week acclimation period.

Experimental Design

A total of 40 rats were divided randomly into two groups. All were injected s.c. with AOM (15 mg/kg body wt) from 1 week after the start of the experiment, once weekly for three successive weeks. Group 1 (20 rats) were fed diet containing 0.015% JTE-522 and group 2 received basal powder diet as a control group throughout the 30 week experiment. In earlier studies, JTE-522 was not found to cause any pathological changes in the colon (Wei et al., 2003), so no JTE-522 alone group was included here.

At the termination of the experiment, all rats were killed under ether anesthesia, the liver and kidneys were taken and weighed, and the colons were excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. All grossly visible colonic lesions were marked on charts to record their locations and facilitate precise histological investigation. To evaluate tumor volume (v), the long (l) and short (w) dimensions (mm) of each tumor mass (length and width) were measured with calipers and calculated according to the equation $v=(l \times w^2)/2$ (Salim et al., 2000). Then tumors were fixed in 10% buffered formalin and processed for histopathological examination and diagnosis according to the criteria described by Ward (1974).

PCNA Immunohistochemistry

PCNA immunohistochemical staining was carried out for normally appearing colonic mucosa in all non-colon tumor rats from each group to evaluate cell proliferation activity using anti-PCNA antibody (PC-10, IgG2a; Dako,USA) with the avidin-biotin complex (ABC) method. Briefly, sections (3 μm thick) of each colonic mucosa were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were then incubated with 10% normal horse serum at room temperature for 30 min to block background staining and then with PCNA antibody diluted 1:500 in Tris (hydroxymethyl) aminomethane-buffered saline overnight. Afterwards, the sections were exposed to biotinylated horse anti-mouse IgG (Vector Labs, Burlingame, CA, USA) for

Table 1. Data for Average Body Weights, and Absolute and Relative Liver and Kidney Weights of Rats

Group	Treatment	No. of survival rat	Body weight (g)		Liver weight		Kidney weight	
			Initial[a]	Final	Absolute (g)	Relative (g)	Absolute (g)	Relative (g)
1	AOM+JTE-522	18	114±4	334±26	9.6±1.3*	2.9±0.2	2.2±0.2*	0.7±0.1
2	AOM	20	114±5	334±17	8.6±0.6	2.4±0.2	2.0±0.2	0.6±0.1

[a]: Values are mean values ± SD.   *: $p<0.05$ compared with G2

Table 2. Incidence, Multiplicity and Volumes of Colon Tumors in Each Group

Group	Treatment	No. of rat	Incidence (%)			Multiplicity			Average tumor volume (mm³)
			Adenoma	Carcinoma	Total tumor	Adenoma	Carcinoma	Total tumor	
1	AOM+JTE-522	20	1[a](5)	8(40)	8(40)	0.05±0.00	0.40±0.50*	0.45±0.60*	60.11±53.40#
2	AOM	20	1(5)	12(60)	12(60)	0.05±0.00	1.00±1.08	1.05±1.10	88.07±144.30

a: Values are number of rats bearing tumors   b: Values are mean ± SD.
*: $p<0.05$ compared with G2   #: 31.7% down compared with G2

30 min. Finally, peroxidase activity in the colonic mucosae cells was visualized by treatment with 0.02% diaminobenzidine. Nuclei were counterstained with hematoxylin. PCNA-positive cells were scored in 10 glands per rat at random.

Statistical Analysis

Data for body weights, food and water consumption, organ to body weight ratios, and PCNA indices were analyzed by one-way analysis of variance (ANOVA), and mean values were compared using the Dunnett's test ($P<0.05$ considered as statistically significant). The Fisher's exact probability test was used for comparisons of incidence and multiplicities of colon tumors in different groups (StatView Software ver.5, Abacus Concepts, CA, USA).

Results

Final Body Weights and Relative Organ Weights, and Others

During the 30-week experimental period, food and water intakes were almost the same in each group. The final body weights, absolute and relative liver and kidneys weights are summarized in Table 1. No clinical signs or effects on body weight gain related to JTE-522 administration were apparent in any of the groups during the experiment. Daily food consumption and water intake of each group showed no significant differences between the groups (data not shown). There are no differences in relative weights of liver and kidneys found between groups although absolute liver weight and absolute kidneys weight in group 1 were significantly higher than in the controls. Two rats in group 1 were found dead before week 30 with intestinal tumors.

Histological Findings in the Colon

Macroscopically, most tumors developed in the colon, mainly in its middle and distal areas, with none in the proximal segment, and a few in the small intestine. They were sessile or psdunculated tumors, and histologically diagnosed as tubular adenomas adenocarcinomas (Fig 2A and 2B) with a high incidence of signet-ring cell carcinomas. Data for the incidences, multiplicities and volumes of colon tumors are summarized in Table 2.

The incidence of total colon tumors (including the carcinomas) in the JTE-522 treated group was lower than in
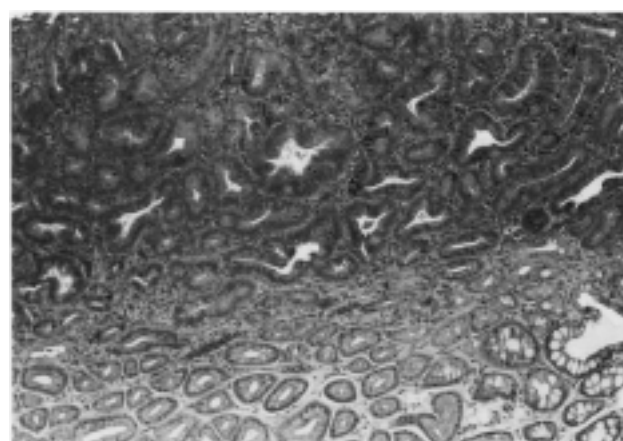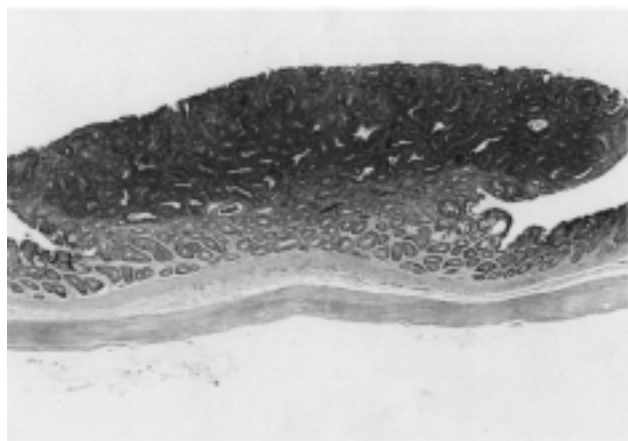

Figure 2. A: Adenocarcinoma of Colon Induced by AOM in a F344 Rat (H&E stainingX40 times). B: Higher Magnification of (A) Showing Glands Lined by Dysplastic Columnar Cells, Goblet Cells, and Paneth Cells with Eosinophilic Apical Cytoplasmic Granules (H&E stainingX 200 times)
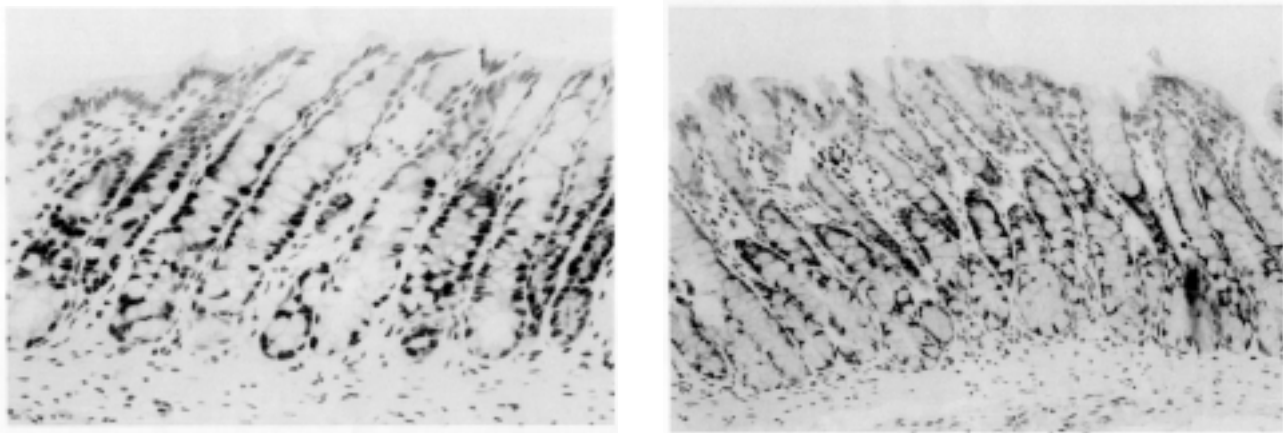
Figure 3. PCNA Immunohistochemistry (X200 times). A: PCNA-labeled Nuclei in Colon Mucosa from a Rat Treated with AOM and JTE-522. B:PCNA-labeled Nuclei in Colon Mucosa from a Rat Treated with AOM only. Note the Decrease in Numbers of PCNA-labeled Nuclei Seen in A Compared with B.

the controls, although this was not significant. A significant reduction was found for multiplicity (including numbers of carcinomas/rat and total tumors/rat) ($P< 0.05$), while the average tumor volume in JTE-522 treated group was 31.7% lowered (not statistically significant).

PCNA-positive Index

Immunohistochemical examination showed that PCNA-labeled nuclei were mostly located in the lower third of the crypts in non-tumorous parts of the colon (Fig.3A and 3B). Fig. 4 illustrates differences in ratios of PCNA-positive nuclei in rat colonic crypts. The JTE-522 treated group was found to have a significantly reduced PCNA-positive index in the mucosal crypts, compared with the control value ($P<0.05$).

Histological Examination of the Other Organs

On gross and microscopic examination of the liver, kidneys and small intestine, a few rats treated with AOM in both groups had mesenchymal renal tumors and/or preneoplastic hepatocellular lesions, and small intestine tumors, but these lesions were found in both treated and control groups, with no obvious influence of the JTE-522 treatment.
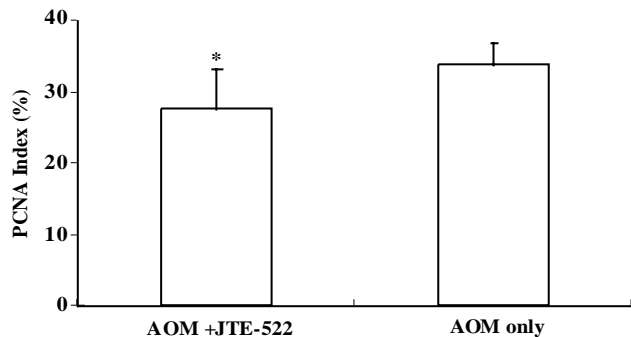

Figure 4. PCNA Index in Non-tumor Colonic Mucosa of Rats Treated with JTE-522.
*: $P<0.05$ compared with control group.

Discussion

The results described here clearly indicate that dietary administration of JTE-522 inhibits AOM-induced colon carcinogenesis in male F344 rats without causing any adverse toxicological side effects. Moreover, this was associated with significantly lowered PCNA-positive indices in non-tumor colonic mucosal crypts. The result provides the clearly evidence that JTE-522 possesses chemopreventive activity against colon carcinogenesis induced by AOM in rats in line with our earlier observations in a 12-week study (Wei et al., 2003). It was similarly found to inhibit tumor development in DMH-treated rats (unpublished results).

Although NSAIDs are highly effective for the relief of inflammatory disease as COX inhibitor, use of non-specific forms is limited by adverse effects, particularly on the gastrointestinal tract and kidneys. Therefore, selective COX-2 inhibitors have been sought to act as a kind of colon tumor inhibitors (Kakizoe, 2003), JTE-522 being one such strongly selective agent. Li et al (2001) earlier found JTE-522 to suppress *N*-nitrosomethylbenzylamine-induced esophageal tumorigenesis in F344 rats (21) and Tomozawa et al (1999) have provided evidence of inhibition of haematogenous metastasis of colon cancer in a mouse model. Two recent studies indicated that JTE-522 can inhibit liver metastasis of colon cancer *in vitro* (Nagatsuka et al., 2003), possibly due to suppressing vascular endothelial growth factor (VEGF) expression (Yamauchi et al., 2003). In addition, JTE-522 has been found to directly inhibit the growth of bile duct carcinoma and gall bladder carcinoma cell lines *in vitro*, suggesting therapeutic effects on hepatobiliary carcinomas (Hayashi et al., 2001).

From the present assessment of PCNA-positive cell indices in colonic mucosa, one possible mechanism for the decrease in colonic tumors by JTE-522 might be through inhibition of cell proliferation. Increase in the PCNA index correlates with a greater risk of developing colon cancer (Kawabata et al., 1999) and in another study, JTE-522 reduced the percentage of BrdU-positive cells in the colonic

treated with DMH (Wei et al., 2003). Increased cell proliferation is generally considered to play an important role in multistage carcinogenesis (Cohen and Ellwein, 1990), including colon tumorigenesis (Lipkin, 1988). To date, various molecular mechanisms have been proposed to be responsible for the antitumor effects of NSAIDs, including inhibition of cell proliferation, angiogenesis and metastasis, induction of apoptosis, and enhanced immunosurveillance (Shiff and Rigas, 1999; Howe et al., 2001). Indeed, more than one mechanism may be involved in NSAIDs' antitumorigenic effects (Oshima and Taketo, 2002). Recently, we found that JTE-522 possesses chemopreventive activity against induction but not progression of DMH-induced rat colon carcinogenesis (unpublished data).

AOM is a metabolite of DMH in rat liver (Wolfer and Frank, 1982). A molecular study found Ki-ras and p53 genes to be mutated in most colon cancers in AOM and DMH treated rats (Erdman et al., 1997). There are many similarities between AOM and DMH induced rat colon carcinogenesis, and future studies may need to be concentrated on chemical bioactivity and absorption in the intestine to explore the chemopreventive mechanisms of JTE-522.

In summary, the present study demonstrated inhibitory effects of JTE-522 on AOM-induced rat colon carcinogenesis, and suggested a possible mechanism—inhibition of cell proliferation in colonic mucosa. Further studies of the antitumorigenic and chemopreventive effects of this compound appear warranted.

Acknowledgements

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