Inhibitory effect of sulindac on DMBA-induced hamster cheek pouch carcinogenesis and its derived cell line

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Abstract. In order to investigate the involvement of cyclo-oxygenase (COX)-2 in oral carcinogenesis and chemo-prevention for it, we examined the COX-2 expression during dimethylbenzanthracene (DMBA)-induced hamster cheek pouch carcinogenesis and the inhibitory effect of sulindac, a non-steroidal anti-inflammatory drug (NSAID), on the carcinogenesis and its derived squamous carcinoma cell line HCPC-1. From the beginning of DMBA application, basal diet or diets containing sulindac 200 or 400 ppm were given to hamsters, and observation of tumor development and measurement of body weight were performed. Immunohisto-chemical analysis revealed that COX-2 expression was increased toward carcinogenesis from epithelial dysplasia to squamous cell carcinoma (SCC). All hamsters developed SCC, but the onset of carcinoma formation was significantly delayed up to 14.8 and 11.8 weeks in the 200 ppm, and 400 ppm sulindac group, respectively, as compared to 8.7 weeks in the control group. In addition, tumor growth was retarded in the group of sulindac treatment, and mean survival time was 23.7 weeks in the control group and 36.3 and 33.8 weeks in the 200 and 400 ppm sulindac group, respectively. Body weight loss was not observed during the experimental period. Histologically, administration of sulindac inhibited angiogenesis in the tumor stroma. Treatment with sulindac sulfide, an active metabolite of sulindac, caused inhibition of cell growth, PGE_2 production and VEGF production in HCPC-1 cells *in vitro*. Expression of COX-2 protein in HCPC-1 cells was also decreased 2-fold by treatment with sulindac sulfide. It was thus indicated that inhibitory effects were partly due to inhibition of tumor angiogenesis by sulindac. These findings suggested the involvement of COX-2 in DMBA-induced hamster cheek pouch carcinogenesis and the chemopreventive potential of sulindac.

Introduction

Epidemiological and laboratory studies have indicated an inverse association between the risk of colorectal cancer and intake of non-steroidal anti-inflammatory drugs (NSAIDs) (1-4). Cyclooxygenases (COXs) have been postulated to be target molecules for NSAIDs and there are two known distinct isoforms in COXs, COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues and appears to be a housekeeping enzyme responsible for various physiological functions. On the other hand, COX-2 is an enzyme induced by mitogens, cytokines and growth factors of epithelial cells, and its pathophysiological role has been primarily connected to prostaglandin (PG) production in response to inflammation (5-8).

Sulindac, an NSAID, has been extensively studied for the pharmacological actions in both humans and animals. When administered orally, sulindac is reduced by gut flora to yield the active anti-inflammatory metabolite, sulindac sulfide, prior to absorption. Sulindac inhibits colorectal carcinogenesis in rodent models (9,10) and causes regression of adenomas (2,11) in patients with familial adenomatous polyposis coli. The mechanism by which this activity occurs is unclear, although some correlation has been found between sulindac treatment and reduced levels of prostaglandins in the human colorectal mucosa (12,13). In relation to reduced prosta-glandin levels, sulindac treatment was not found to be chemo-preventive in all studies (2,13). In addition, several studies indicate that sulindac and its metabolites have demonstrated chemoprotective effects through inhibition of angiogenesis (14,15).

In this study, we examined the COX-2 expression during DMBA-induced hamster cheek pouch carcinogenesis and the inhibitory effect of sulindac on the carcinogenesis via inhibition of angiogenesis.

Materials and methods

Animals and carcinogen treatment. Eighteen male golden Syrian hamsters (5-week old) were purchased from Nihon Animal, Inc., Osaka, Japan and were treated by painting

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a buccal pouch 3 times a week for 13 weeks with a 0.5% solution of 9,10-dimethyl-1, 2-benzanthracene (DMBA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in acetone as described before (16). From the beginning of DMBA application, basal diet CE-2 (Japan Clea Co., Ltd., Tokyo, Japan) was given to hamsters and tumor development was observed. At intervals, tissue specimens applied with DMBA were taken for the histological and immunohisto-chemical examinations. Animals were sacrificed under ether anesthesia at the 10, 13 and 16th week after the beginning of DMBA application.

Histological and immunohistochemical studies. Tissue specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm thick sections according to conventional procedures. Histological examination was performed by hema-toxylin and eosin (H&E) stain. For immunohistochemical studies, a modification of the streptavidin-biotin-peroxidase-complex (SABC) method (17) was employed by using rabbit polyclonal antibody against human COX-2 (IBL, Gunma, Japan). Non-immunized rabbit serum was used as negative control.

Administration of sulindac. From the beginning of DMBA application, hamsters were fed with basal diet or diets containing 200 and 400 ppm sulindac (Sigma-Aldrich Co., St. Louis, MO, USA). DMBA application was continued until the 13th week and observation of tumor development and measurement of body weight were performed. The relative tumor weights (mg) were calculated using the formula a^2 x b/2, where a is the width in millimeters and b is the length in millimeters, according to the method of Battelle Columbus Laboratories (18).

Cell line and cell culture. HCPC-1 (19) cells were isolated and established from 7,12-dimethylbenz (α) anthracene-transformed Syrian golden hamster cheek pouch squamous cell carcinoma and kindly provided by Professor G. Shklar (Harvard School of Dental Medicine, Boston, MA, USA). The culture was maintained in Dulbecco's modified Eagle's MEM (DMEM, Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone Labo-ratories, Logan, UT, USA) and 4 mM L-glutamine as growth medium at 37˚C in a 5% CO_2 incubator.

Cell growth assay. Cells were plated at 2.5×10^4 cells/ml in dishes and cultured in growth medium at 37˚C. Sulindac sulfide (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) (Dojindo Laboratories, Kumamoto, Japan) before use and added to the culture medium at various concen-trations after 24-h cultivation of cells. After further incubation at 37˚C for 72 h, cell growth was assessed by cell count using by trypan blue dye exclusion test by conventional microscopy. The IC_{30} concentration of sulindac sulfide was chosen as a less cytotoxic dose in a preliminary experiment.

Immunoassay for prostaglandin E_2 (PGE$_2$) and vascular endothelial growth factor (VEGF). HCPC-1 cells were plated at 5×10^4 cells in 3.5 ml growth medium in 60-mm tissue culture dish and grown at 37˚C for 24 h. The IC_{30} concen-tration of sulindac sulfide was added to the dishes and then cells were cultured for further 72 h. The medium was discarded and 3.5 ml of serum-free fresh medium was added to each dish, and then conditioned medium was collected after 15 min for PGE$_2$ assay and after 24 h for VEGF assay. PGE$_2$ assay was performed as described in a protocol of prostaglandin E$_2$ EIA kit (Cayman Chemical, Ann Arbor, MI, USA) and VEGF assay was performed as described in a protocol of Quantikine Human VEGF Immunoassay (R&D systems, McKinley, MN, USA).

Assay for microvessel density. Microvessels were detected by immunohistochemical staining for factor VIII/von Willebrand factor, a marker for vascular endothelial cells. After pre-treatment with 0.25% trypsin for 10 min, tissue sections were immunostained with a rabbit-factor VIII antibody (1:800; Dako Corporation, Carpinteria, CA, USA) using the SABC method. Microvessel density (MVD) was determined by counting the number of vessels in high magnification field (x400) of the tumor stroma that contained the highest number of capillaries.

Western blot analysis. Cell lysates were subjected to Western blot as described previously (20). The primary antibodies used were goat polyclonal antibodies against COX-2 and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were anti-goat IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology). Actin was used as an internal control.

Statistical Analysis. Statistical analysis was done by using the Student's t-test and Fisher's exact test. Differences were considered significant when the p-value was <0.05.

Results

Increased COX-2 expression during DMBA-induced hamster cheek pouch carcinogenesis. All hamsters were successfully treated 3 times a week with a 0.5% solution of DMBA dissolved in acetone and developed papillomatous tumors including precancerous or cancerous lesions. Approximately at the 5th week after DMBA application to buccal pouch, these hamsters showed hyperorthokeratosis of the buccal pouch squamous epithelium. From the 6th to the 9th week after DMBA application, papillomatous tumors developed in all hamsters. Histologically, these tumors resembled that of the squamous papilloma with or without mild epithelial dysplasia which was characterized by cellular atypia of the basal cells and some parabasal cells, mild nuclear pleomor-phism and hyperchromatism. Additional topical application with DMBA induced a neoplastic transformation and formed carcinoma *in situ* or early invasive SCC. Finally, invasive SCC was produced by the 10th week after DMBA application and grew rapidly.

Immunohistochemical analysis revealed that COX-2 expression was increased toward carcinogenesis from epithelial dysplasia to SCC (Fig. 1). Normal squamous epi-thelium showed no COX-2 expression in the epithelial cells. In contrast, epithelial dysplasia showed positive reaction for COX-2 protein in basal cells and some parabasal cells.
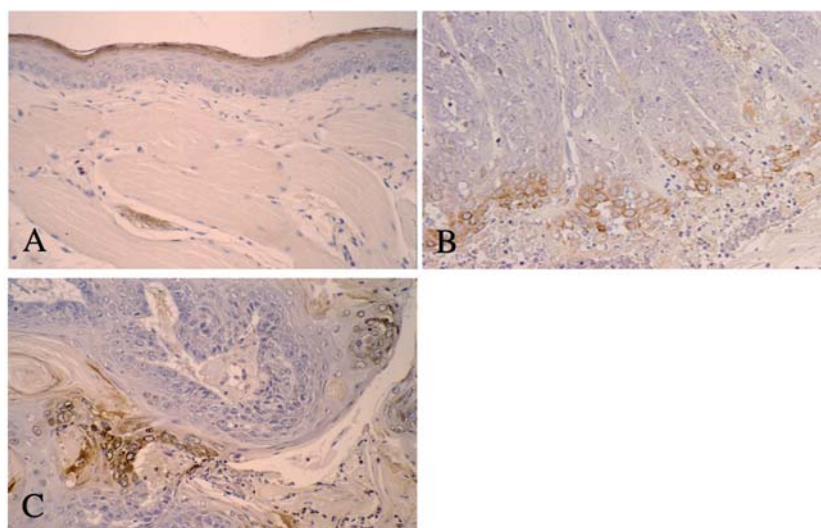
Figure 1. Photomicrographs of DMBA-induced SCC in hamster cheek pouch epithelium and immunohistochemical expression of COX-2 protein in the process of carcinogenesis. (A) Normal epithelium, (B) epithelial dysplasia, and (C) well-differentiated SCC in immunohistochemical staining for COX-2. (A-C) Original magnification, x200.

Table I. Chemopreventive activity of sulindac against DMBA-induced hamster cheek pouch carcinogenesis.

	Tumor incidence (%)	Onset of carcinoma formation (weeks)	Duration until relative tumor weight reached 500 mg from onset (weeks)	Body weight at 13th week (g)	Mean survival time (weeks)
Control (n=6)	6/6 (100)	8.7±1.5	5.2±1.2	140.7±18.4	23.7±2.5
Sulindac 200 ppm (n=6)	6/6 (100)	14.8±0.75[a]	6.5±3.3	146.0±15.3	36.3±10.6[b]
Sulindac 400 ppm (n=6)	6/6 (100)	11.8±0.75[a]	6.8±1.8	159.3±15.2	33.8±5.1[a]

Hamsters (5-week old) were treated by painting a buccal pouch 3 times a week for 13 weeks with a 0.5% DMBA solution dissolved in acetone. From the beginning of DMBA application, basal diet or diets containing sulindac 200 and 400 ppm were given to hamsters, and observation of tumor development and measurement of body weight were performed. Values presented are mean ± SD. [a]$p<0.005$; [b]$p<0.05$.
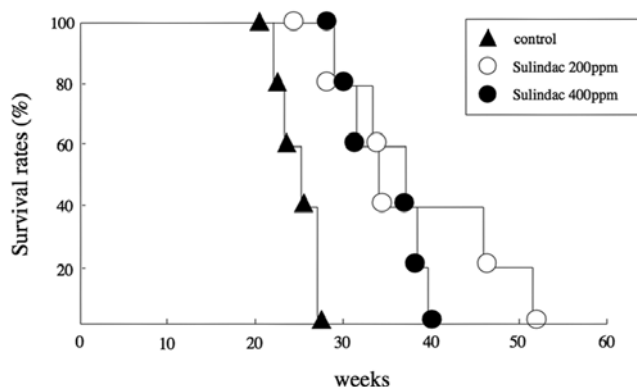

Figure 2. Survival time of hamsters with DMBA-induced cheek pouch SCC. ▲, basal diet; ○, diets containing sulindac 200 ppm; ●, diets containing sulindac 400 ppm; n=6 in each group.

Furthermore, early invasive SCCs (EISCC) and invasive SCCs demonstrated moderately or strongly positive for COX-2. In all cases, COX-2 expression was observed in the perinuclear region to the cytoplasma.

Inhibitory effect of sulindac on tumor development and growth. Administration of sulindac was found to have a chemopreventive action against DMBA-induced cheek pouch carcinogenesis. Although all hamsters developed SCC, the onset of carcinoma formation was delayed significantly; 8.7 weeks in the control group and 14.8 and 11.8 weeks in the 200 ppm, and 400 ppm sulindac group, respectively. In addition, tumor growth was retarded in the groups of sulindac treatment. The body weights were similar in the 200 ppm sulindac group or rather increased in the 400 ppm sulindac group as compared to the control group (Table I). Mean survival time was markedly prolonged; 23.7 weeks in the control group, and 36.3 and 33.8 weeks in the 200 and 400 ppm sulindac groups, respectively (Fig. 2).

Inhibition of cell growth, PGE$_2$ and VEGF production in HCPC-1 cells by sulindac sulfide. Sulindac sulfide caused growth inhibition of HCPC-1 cells in a dose-dependent
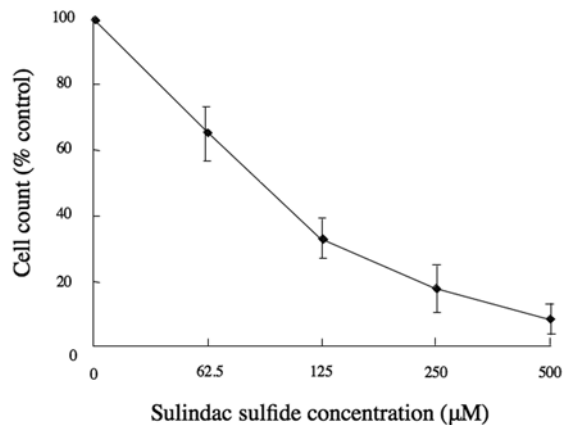
Figure 3. Cell growth inhibition in HCPC-1 cells by sulindac sulfide. Cell viability was determined by cell count after treatment of cells with various concentrations of sulindac sulfide at 37°C for 72 h. Data represent the average ± SD in triplicate dishes.
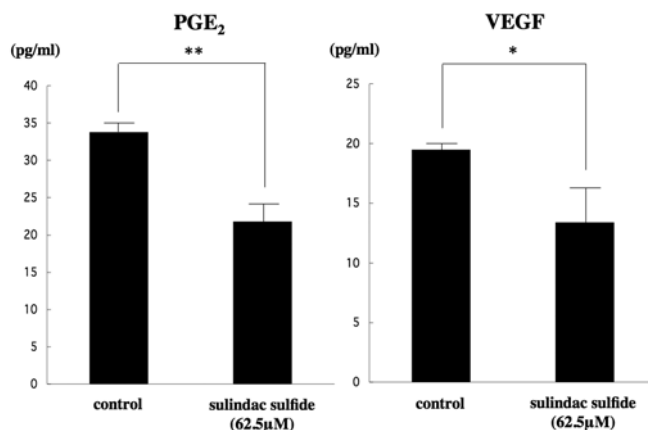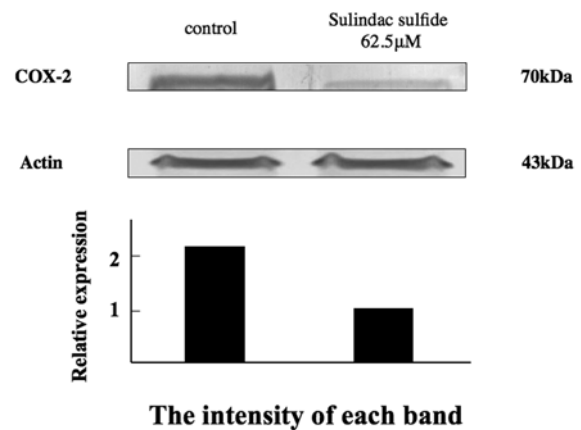

Figure 5. Western blot analysis of COX-2 protein before and after sulindac sulfide treatment in HCPC-1 cells. Expression levels of COX-2 in HCPC-1 cells were decreased by treatment 62.5 μM sulindac sulfide at 37°C for 72 h.

Figure 4. Effect of sulindac sulfide on PGE$_2$ and VEGF production in HCPC-1 cells. HCPC-1 cells were plated at 5×10^4 cells in 3.5 ml growth medium in 60-mm tissue culture dishes and grown at 37°C for 24 h. The IC$_{30}$ concentrations of sulindac sulfide were added to the dishes and then cells were cultured for further 72 h. The medium was discarded and 3.5 ml of serum-free fresh medium was added to each dish, and then conditioned medium was collected after 15 min for PGE$_2$ assay and after 24 h for VEGF assay. Data represent mean ±SD of 3 determinations. *P<0.05, **P<0.005.
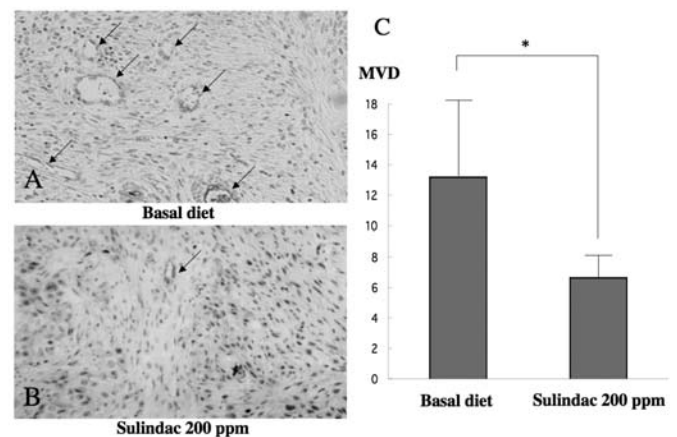

Figure 6. Microvessel formation in the stroma of DMBA-induced SCC and its inhibition by sulindac. Immunohistochemical staining for factor VIII/von Willebrand factor performed to detect microvessels in the tumor stroma of hamster cheek pouch, given (A) basal diet or (B) diets containing 200 ppm sulindac. Arrows, microvessels (original magnification, x100). (C) Microvessel density (MVD) of each group (n=3). MVD was determined by counting the number of vessels in high-magnification field (x400) of the tumor stroma that contained the highest number of capillaries. *P<0.05.

manner (Fig. 3). Treatment with sulindac sulfide at the IC$_{30}$ concentration of 62.5 μM for 3 days suppressed significantly PGE$_2$ and VEGF production by 64 and 69% of the control level in HCPC-1 cells (Fig. 4).

Decreased expression of COX-2 by sulindac sulfide. The expression of COX-2 in untreated cells was compared with that in sulindac sulfide-treated cells by Western blotting. Expression of COX-2 protein in HCPC-1 cells by treatment with 62.5 μM sulindac sulfide was decreased by 2-fold (Fig. 5).

Inhibition of tumor angiogenesis by sulindac. When the effect of sulindac on tumor-induced angiogenesis in tumor stroma was examined histologically, the MVD was significantly lower in sulindac groups than in the control group

given basal diet (Fig. 6). The effect was more prominent in the 200 ppm group than in the 400 ppm group.

Discussion

Cyclooxygenases (COXs) catalyzes the rate-limiting step in prostaglandin (PG) biosynthesis; the conversion of arachidonic acid to the PG endoperoxides PGG$_2$ and PGH$_2$. They have been postulated to be target molecules for NSAIDs. Numerous experimental rodent models as well as recent epidemiological studies have shown an inverse relationship between NSAIDs intake and colon cancer development (5,6,21-23). Although the precise mechanism by which NSAIDs inhibit carcinogenesis is unclear, the available data support the hypothesis that arachidonic acid metabolism is altered through COX activity, thereby reducing eicosanoid production (24). Several experimental animal and human

colon tumors contain high levels of PGs, which have been shown to affect cell proliferation and tumor growth and suppress immune responsiveness (25,26). It is therefore reasonable to predict that inhibition of colonic mucosal or tumor PGs by specific agents may suppress the tumorigenicity of colon. The increased level of COX-2 activity may result in elevated PG levels in these lesions. COX-2 expression has also been shown to be up-regulated in human SCC of the head and neck region including oral mucosa (27). Similar findings were obtained in the rat tongue SCC model induced by 4NQO (28). The results obtained in the present study demonstrated that increased levels of COX-2 protein are present not only in oral SCC but even in epithelial dysplasia. These data indicated that administration of sulindac produced inhibitory effects against oral SCC development both at the initiation phase and during promotion and progression phases of carcinogenesis.

Administration of NSAIDs such as aspirin and indomethacin is associated with gastrointestinal ulceration, because of their COX-1 inhibitory actions (23), and to date most studies have implicated COX-2, rather than COX-1, as an isoform involved in carcinogenesis. In the azoxymethane model of rat colon carcinogenesis, Reddy *et al* (23) found that 1500 ppm celecoxib inhibits aberrant crypt foci multiplicity by 40-49% without gross changes in the intestines. We have also reported that celecoxib inhibited DMBA-induced hamster cheek pouch carcinogenesis in a dose-dependent manner (29).

In the present study, the body weights were similar or rather increased in the sulindac group as compared to the control group, indicating no side effect. In addition, there seems to be an optimal dose of sulindac to inhibit carcinogenesis, because 200 ppm of sulindac was more effective for inhibition of tumor development and growth, and survival time than 400 ppm of sulindac. Thus, it was suggested that sulindac is a potent and non-toxic chemoprotective agent.

In recent years, the correlation between angiogenesis and development of tumor can be seen clearly in many reports, an adequate blood flow in tumor stroma was indeed essential for growth of tumors >1-2 mm in size (30). It has also been assumed that transmissive, excessive aberrant new blood vessels contribute to metastasis. PGE$_2$, which is a main product of COX-2, promotes angiogenesis with inflammatory granulation tissue (31). Expression of angiogenesis factors such as VEGF and TGF-ß is found in colorectal cancer cell lines which overexpress COX-2, and a production of angiogenesis factor was inhibited dose-dependently by COX-2 inhibitor (32). In intestinal polyps occurring in APC knockout mice, COX-2 is induced by interstitial cells and produced PGE$_2$ induces VEGF via an EP$_2$ receptor, resulting in new angiogenesis in tumor tissues (33). In this study, production of VEGF was significantly inhibited by sulindac sulfide *in vitro*, and the MVD was significantly decreased when animals were fed diets containing sulindac *in vivo*. Therefore, it was thought that angiogenic inhibition was important as one of the mechanisms of anti-tumor effect of NSAIDs.

In conclusion, our present results clearly indicated that COX-2 protein is highly expressed in dysplastic precancerous lesions and SCCs, and provided evidence that sulindac, a NSAID, possesses chemopreventive potential against DMBA-induced hamster cheek pouch carcino-genesis via anti-angiogenic action. Based on these findings, it is suggested that sulindac could serve as a potent chemopreventive agent with low toxicity against oral carcinogenesis.

Acknowledgments

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