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Increased expression of cyclooxygenase (COX)-2 in DMBA-induced hamster cheek pouch carcinogenesis and chemopreventive effect of a selective COX-2 inhibitor celecoxib

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BACKGROUND: In recent years, overexpression of cyclooxygenase (COX)-2 protein and mRNA has been reported in various cancer tissues. Therefore, it has been suggested that COX-2 is related to carcinogenesis.

METHODS: Hamsters were treated by painting a buccal pouch with a 0.5% DMBA solution dissolved in acetone. Basal diet or diets containing 150, 500 and 1500 ppm of celecoxib, a selective COX-2 inhibitor, were given *ad libitum* to hamsters, and tumor development was observed.

RESULTS: Immunohistochemical and Western blot analyses revealed that COX-2 expression was increased toward the carcinogenesis. Although all hamsters developed squamous cell carcinoma, the onset of tumor formation was delayed in a dose-dependent manner. Also, tumor growth was retarded and survived animals were increased in the group of celecoxib treatment. Histologically, administration of celecoxib increased the apoptotic cells in the tumor parenchyma and significantly inhibited the angiogenesis in the stroma.

CONCLUSIONS: The COX-2 expression was increased during hamster cheek pouch chemical carcinogenesis. Administration of celecoxib demonstrated the chemopreventive potential against the carcinogenesis.

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Keywords: celecoxib; chemoprevention; cyclooxygenase-2; DMBA; hamster cheek pouch carcinogenesis

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Introduction

Cyclooxygenase (COX) catalyzes the rate-limiting step in prostaglandin (PG) biosynthesis; the conversion of arachidonic acid to the PG endoperoxides PGG_2 and PGH_2. There are two different isoforms of COX, COX-1 and COX-2. They have been postulated to be target molecules for non-steroidal anti-inflammatory drugs (NSAIDs). COX-1 is constitutively expressed in most normal tissues and appears to be a housekeeping enzyme responsible for various physiological functions, such as cytoprotection in the stomach, vasodilation in the kidney, and production of the proaggregatory prostanoid thromboxane by platelets (1). However, COX-2 is an enzyme induced by mitogens, cytokines and growth factors of epithelial cells, and its pathophysiological role has been primarily connected to PG production in response to inflammation (2–5). Epidemiological and laboratory studies indicated an inverse association between the risk of colorectal cancer and intake of NSAIDs such as aspirin, indomethacin and sulindac (6–9). In recent years, overexpression of COX-2 protein and mRNA has been reported in various cancer tissues such as colon, stomach, breast, lung, esophagus, pancreas, endocervix, urinary bladder, prostate and skin (10–19). In head and neck squamous cell carcinoma (SCC), several investigators have shown that expression of COX-2 is up-regulated, but its significance in relation to tumor development and growth is unclear (20, 21).

Oral cancer is one of 10 most frequent cancers in the world. SCC is the most common malignant tumor of the oral cavity, accounting for over 90% of the malignant neoplasms in this region, and is thought to arise from a progressive dysplasia of overlying oral squamous epithelium. Furthermore, recent epidemiologic data have indicated that the incidence of oral cancer is increasing. Therefore, new molecular targets

are needed for the prevention and treatment of oral cancer.

In this study, we examined the COX-2 expression during DMBA-induced hamster cheek pouch carcinogenesis and the chemopreventive effect of celecoxib, a selective COX-2 inhibitor, for tumor development and growth.

Materials and methods

Animals and carcinogen treatment

Seventeen male golden Syrian hamsters (5-week old) were purchased from Nihon Animal, Inc., Osaka, Japan and were treated by painting a buccal pouch three 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 or with acetone alone as the control as described before (22). From the beginning of DMBA application, basal diet CE-2 (Japan Clea Co., Ltd., Tokyo, Japan) were given to hamsters, and tumor development was observed. At intervals, tissue specimens applied with DMBA or with acetone alone were taken for the histological and immunohistochemical examinations and Western blot analysis. Animals were sacrificed under ether anesthesia at the 10, 13 and 16th week after the beginning of DMBA application.

Histological and immunohistochemical studies

The tumor tissues were fixed in 10% formalin, embedded in paraffin and cut into 4 μm thick sections. The sections were stained with hematoxylin and eosin (H & E) for histological examination. For immunohistochemical examination, a modification of the streptavidin-biotin-peroxidase-complex (SABC) method (23) was used. The tissue sections were deparaffinized and rehydrated in a graded series of alcohols. The sections were microwaved three times for 5 min each in citrate phosphate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 15 min. The section were incubated with 10% normal bovine serum albumin in phosphate-buffered saline (PBS) for 10 min to block non-specific background staining. Monoclonal antibody against COX-2 (C22420; Transduction Laboratories, Lexington, KY, USA) was applied as a primary antibody at a dilution of 1:50 at 4°C overnight. Then, the streptavidin-biotin complex (LSAB-2; Dakopatts, Copenhagen, Denmark) immunoperoxidase reaction was carried out for 1 h at 4°C. After washing with PBS, biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) was applied to the sections, which were then incubated for 30 min at room temperature. Peroxidase reactivity was detected with the use of 3,3′-diaminobenzidine tetrahydrochloride containing 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, dehydrated and mounted with glycerol gelatin.

Western blot analysis

Tissue specimens were frozen in liquid nitrogen and stored at −80°C. The frozen tissues were homogenized on dry-ice and were lysed in a lysis buffer composed of PBS supplemented with 20 mM Tris-HCl, pH 8.0, 1% NP40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% β-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase inhibitors including 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM benzamidine, 1 μg/ml pepstatin, 2 μg/ml antipain hydrochloride (Boehringer, Mannheim, Germany), 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Wako Pure Chemical), 2 mM sodium orthovanadate (Sigma-Aldrich Co., St Louis, MI, USA), and 20 U/ml ulinastatin (Mochida Pharmaceutical, Tokyo, Japan). The lysate containing 15 μg of protein was electrophoresed in a 10–20% gradient SDS-PAGE mini gel (Dai-ichi Kagaku, Tokyo, Japan) and blotted onto a PVDF membrane using Multiphor II (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 30 min. The blotted membrane was blocked with 5% skim milk in 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl and 0.5% Tween 20, and incubated with primary antibodies (0.1–1 μg/ml) described below for 16 hr at 4°C. The membrane was then incubated with alkaline phosphatase-conjugated secondary antibodies (0.02 μg/ml) described below for 4 hr at room temperature. The membrane was rinsed and treated with nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) to visualize protein bands. The primary antibodies used were mouse monoclonal antibody against COX-2 (Transduction Laboratories) and goat polyclonal antibodies against actin (Santa Cruz, Santa Cruz, CA, USA). The secondary antibodies used were anti-mouse, or -goat IgGs conjugated with alkaline phosphatase (Santa Cruz).

Administration of celecoxib

From the beginning of DMBA application, 24 hamsters (six hamsters per group) were fed *ad libitum* with basal diet or diets containing 150 ppm (0.15 mg/g diet), 500 ppm (0.5 mg/g diet) and 1500 ppm (1.5 mg/g diet) celecoxib (Pharmacia Co Ltd. Tokyo, Japan). 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 a formula of $a^2 \times b/2$, where a is the width in millimeters and b is the length in millimeters, according to the method of Battelle Columbus Laboratories (24). At the 26th week, survived hamsters were sacrificed and tissue specimens applied with DMBA were taken for the histological and immunohistochemical examinations.

Assay for tumor angiogenesis

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 anti-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-

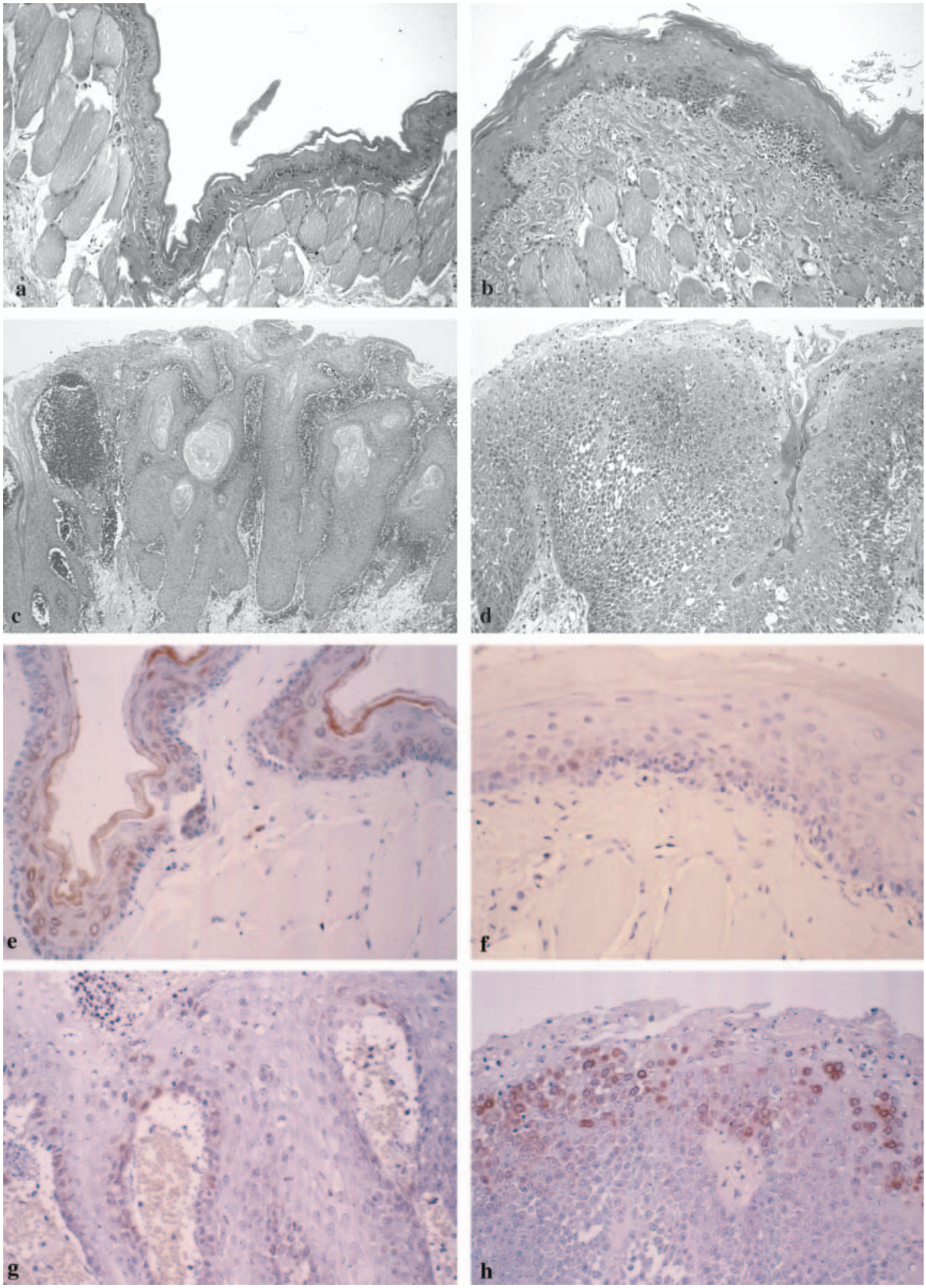

Figure 1 Photomicrographs of DMBA-induced tumors in hamster buccal pouch epithelium and immunohistochemical expression of COX-2 protein in the process of carcinogenesis. (a, e) acetone-treated epithelium, (b, f) epithelial dysplasia, (c, g) early invasive SCC, and (d, h) well-differentiated SCC in H & E staining (a–d, original magnification ×100) and immunohistochemical staining for COX-2 (e–h, original magnification ×200).

magnification field (×400) of the tumor stroma that contained the highest number of capillaries.

The MVD of different groups was compared by the unpaired Student's *t*-tests. Differences with calculated P values < 0.05 were regarded as significant.

TUNEL method

To detect DNA breaks, *in situ* terminal deoxynucleotidyl transferase (TdT) mediated dUTP-digoxigenin nick-end labeling (TUNEL) was performed according to the method of Gavrieli et al. (25). Briefly, after deparaffinization and blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol for 30 min at room temperature, the sections were treated with 20 μg/ml proteinase K (DAKO) for 15 min at room temperature. The sections were submitted to TdT reaction containing terminal transferase and biotin-16-dUTP for 60 min at 37°C. Incubation with diluted peroxidase-conjugated streptavidin for 30 min at room temperature was employed for the detection of biotin-16-dUTP labeling, followed by color development with a solution containing 3,3′-diaminobenzidine and H_2O_2. Methylgreen was used for counterstaining. The apoptotic index (AI; percentage of apoptotic cells) was obtained as the ratio of the number of TUNEL-positive cancer cells to tumor cells counted in the well-labeled areas, as determined by scanning at low magnification.

Results

Increased COX-2 expression during DMBA-induced hamster cheek pouch carcinogenesis

All 17 hamsters were successfully treated three times a week with a 0.5% solution of DMBA dissolved in acetone and developed papillomatous tumors including pre-cancerous 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 pleomorphism and hyperchromatism. Additional topical application with DMBA induced a neoplastic transformation and formed carcinoma *in situ* or early invasive SCC (EISCC). Finally, invasive SCC was produced by the 10th week after DMBA application and grew rapidly. On the other hand, the buccal pouches treated with acetone alone showed epithelial hyperplasia with chronic inflammatory change in the subepithelial region (Fig. 1).

Immunohistochemical and Western blot analyses revealed that COX-2 expression was increased toward the carcinogenesis from epithelial dysplasia to SCC (Fig. 1). Normal squamous epithelium 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. Furthermore,

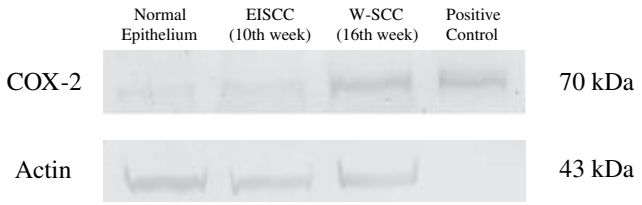

Figure 2 Western blot for COX-2 protein in DMBA-induced tumor tissue specimens. EISCC, early invasive SCC; W-SCC, well-differentiated SCC. Positive control of COX-2 protein was provided together with monoclonal antibody from Transduction Laboratories (Lexington, KY, USA). Actin was used as an internal standard.

EISCCs and invasive SCCs demonstrated moderately or strongly positive for COX-2 not only in basaloid tumor cells but also in other tumor cells. In the epithelial hyperplasia treated with acetone alone as the control, basal cells were weakly positive occasionally. In all cases, COX-2 expression was localized in the nuclear membrane. Western blot analysis demonstrated that COX-2 protein band was more prominent in well differentiated SCC (W-SCC) than that of normal epithelium or EISCC, supporting the immunohistochemical findings (Fig. 2).

Chemopreventive effect of celecoxib

Administration of celecoxib was found to have a chemopreventive action against DMBA-induced cheek pouch carcinogenesis. Although all 24 hamsters developed SCC, the onset of carcinoma formation was delayed in a dose-dependent manner; 8.7 weeks in the control group and 8.7, 10.0 and 11.2 weeks in the 150, 500 and 1500 ppm celecoxib group, respectively. In addition, tumor growth was retarded in the celecoxib group without the body weight loss. The body weights were rather increased in the celecoxib group as compared with the control group (Table 1). At the 26th week, survived animals were one of six in the control group and five of six, five of six and six of six in the 150, 500, and 1500 ppm celecoxib groups, respectively.

Inhibition of tumor-induced angiogenesis and induction of apotosis by celecoxib

When the effect of celecoxib on tumor-induced angiogenesis in tumor stroma was examined histologically, the MVD was significantly lower in the celecoxib groups than in the control group given basal diet ($P < 0.005$). The effect was most prominent in the 150 ppm group (Fig. 3). On the contrary, when apoptosis induction in tumor parenchyma by celecoxib was examined by TUNEL method, apoptotic cancer cells identified by brown nuclear TUNEL signals were clearly observed in the celecoxib groups but not in the control group (Fig. 4). Mean AI was $< 1\%$ in each group, but the reactivity was increased in a dose-dependent manner. In the group administered with 1500 ppm of celecoxib, the TUNEL-positive cells were seen as discrete foci (Fig. 4).

Table 1 Chemopreventive activity of celecoxib against DMBA-induced hamster cheek pouch carcinogenesis

	Tumor incidence (%)	*Onset of carcinoma formation (weeks)*	*Duration until relative tumor weight was reached to 500 mg from onset (weeks)*	*Body weight at 13th week (g)*	*Survived animals at 26th week (%)*
Control ($n = 6$)	6/6 (100)	8.7 ± 1.5	5.2 ± 1.2	147.0 ± 18.4	1/6 (17)
Celecoxib 150 ppm ($n = 6$)	6/6 (100)	8.7 ± 0.52	6.3 ± 1.6	156.3 ± 20.3	5/6 (83)
Celecoxib 500 ppm ($n = 6$)	6/6 (100)	10.0 ± 1.5	6.0 ± 1.4	167.3 ± 10.8	5/6 (83)
Celecoxib 1500 ppm ($n = 6$)	6/6 (100)	11.2 ± 1.6*	6.2 ± 0.75	177.3 ± 18.2*	6/6 (100)

Hamsters (5-week old) were treated by painting a buccal pouch three 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 celecoxib 150, 500 and 1500 ppm were given to hamsters, and observation of tumor development and measurement of body weight were performed. Values presented are each mean ± SD. *$P < 0.05$ in Student's *t*-test.

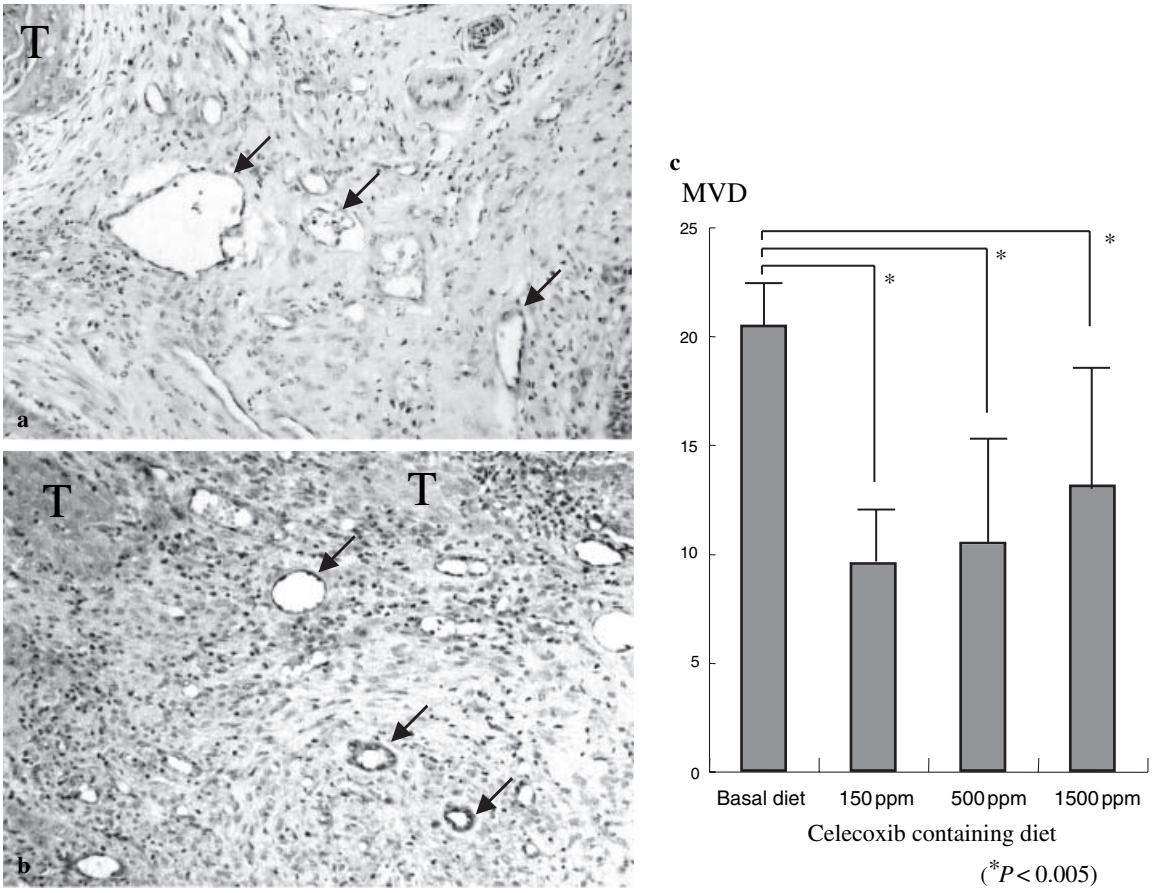

Figure 3 Microvessel formation in the stroma of DMBA-induced SCCs and its inhibition by celecoxib. Immunohistochemical staining for factor VIII/von Willebrand factor was performed to detect microvessels in the tumor stroma of hamster cheek pouch, given (a) basal diet or (b) diets containing 150 ppm celecoxib. T, tumor nest, Arrows, microvessels (original magnification ×100). (c) Microvessel density (MVD) of each group ($n = 12$). MVD was determined by counting the number of vessels in high-magnification field (×400) of the tumor stroma that contained the highest number of capillaries.

Discussion

Numerous experimental rodent models as well as recent epidemiological studies have shown an inverse relationship between NSAIDs intake and colon cancer development (26–30). Although the precise mechanism by which NSAIDs inhibits carcinogenesis is unclear, the available data support the hypothesis that arachidonic acid metabolism is altered through COX activity, thereby reducing eicosanoid production (31). Accumulated evidences suggested that PGs have an important role in the pathogenesis of colon cancer because they affect a number of signal transduction pathways that modulate cellular adhesion and growth (32–34), and are involved in proliferation and metastasis (35). One of the most striking events in which PGs have been

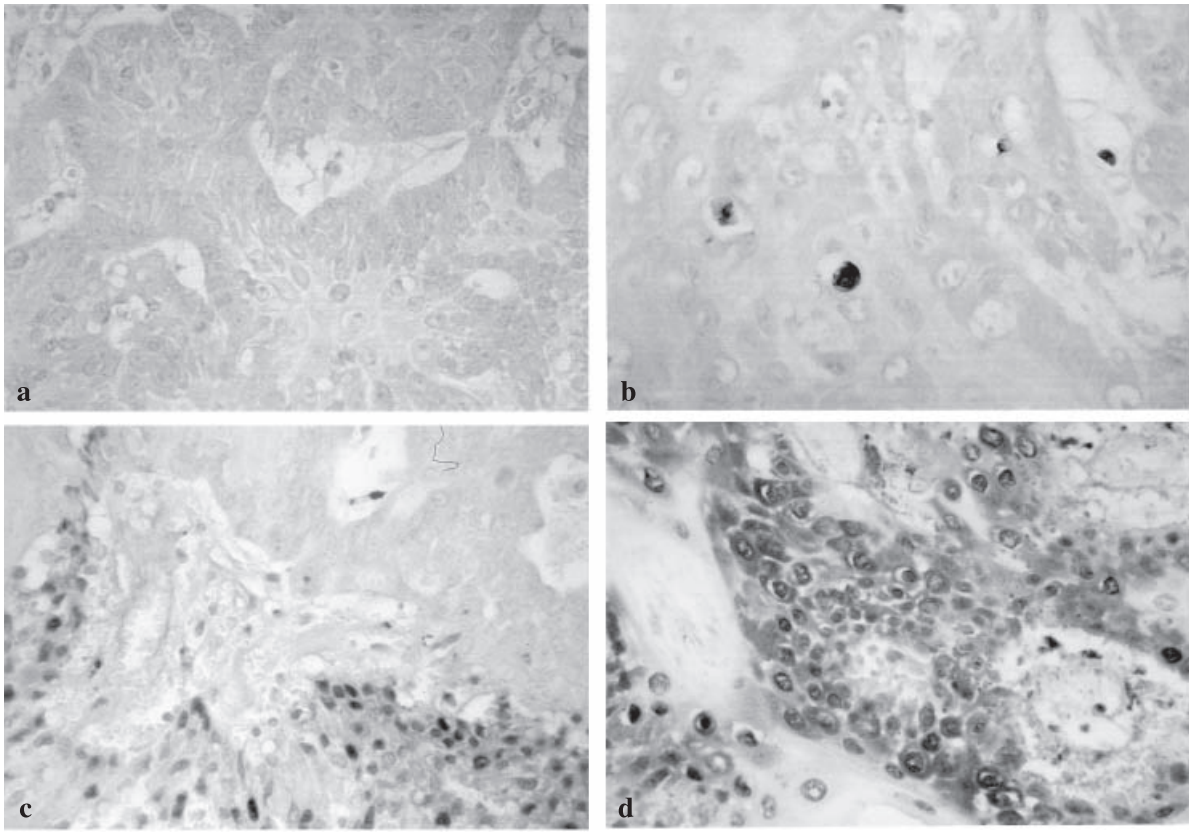

Figure 4 TUNEL staining in the DMBA-induced SCCs of hamster cheek pouch, given (a) basal diet or diets containing (b) 150, (c) 500 and (d) 1500 ppm celecoxib (original magnification ×400).

implicated in tumorigenesis is the inhibition of apoptosis. Apoptosis was shown to be induced by NSAIDs in the colonic epithelium of familial adenomatous polyposis patients (36). Also, we have already reported that celecoxib induced apoptosis in cultured head and neck cancer cell lines to a significantly greater degree than sulindac (37). More directly, Tsujii and DuBois (34) showed that rat intestinal cells increasing COX-2 expression by gene transfer became resistant to butyrate-induced apoptosis, which can be overcome by addition of the non-specific COX inhibitor sulindac sulfide. Several studies have demonstrated the role of COX-2 metabolites, particularly PGE_2, in colon tumor promotion (31, 38). The increased level of COX-2 activity may result in elevated PG levels in these lesions. COX-2 expression has recently been shown to be up-regulated in human SCC of the head and neck region including oral cavity (20). This was also found in the rat tongue SCC model induced by 4NQO (21). The results obtained in the present study demonstrated that COX-2 protein are increased not only in oral SCC but also even in epithelial dysplasia and hyperplasia with chronic inflammatory change treated with acetone alone as the control. These data may indicate that administration of celecoxib 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 (30). The development of selective COX-2 inhibitors, such as NS-398 and celecoxib, offers the advantage of retaining COX-1 activity while inhibiting COX-2 (39). In the azoxymethane model of rat colon carcinogenesis, Reddy et al. (30) found that 1500 ppm celecoxib inhibits aberrant crypt foci multiplicity by 40–49% without gross changes in the intestines. Kawamori et al. (40) also reported that celecoxib significantly inhibits both incidence and multiplicity of colon tumors and that the extent of inhibition is greater than or equal to that seen in earlier studies with aspirin, ibuprofen, sulindac, and piroxicam. Long-term administration of celecoxib with 1500 ppm did not induce any toxic side-effects such as body weight loss. It is noteworthy for clinical application that a pilot endoscopic study showed no difference in gastroduodenal mucosa damages between celecoxib group and placebo group (41). Recently, Kazanov et al. (42) have reported that celecoxib inhibits the growth of transformed enterocytes without affecting the growth of normal cells *in vitro*. Therefore, celecoxib could be a very potent and non-toxic chemoprotective agent.

Accumulated evidences suggested that celecoxib is a potent anti-angiogenic agent (43). In the animal model, celecoxib has significant inhibitory activity to tumor angiogenesis (44). In a rat corneal model described by Kenyon et al. (45), COX-2 caused a substantial reduction in the number and length of sprouting

capillaries, with maximal inhibitory activity of 80%. Interestingly, there is evidence to indicate that the expression of COX-2 in cancer was mainly restricted to the new blood vessels, the pre-existing vasculature adjacent to the primary tumor, and the blood vessels invading the metastatic lesion, but the expression was not present in the tumor cells themselves (44). The current study supported the anti-angiogenic effect of celecoxib on tumor angiogenesis, which was attainable at the low levels of dosage. On the contrary, the frequency of apoptotic cancer cells was quite low but became higher in a dose-dependent manner. Although the survival rate at the 26th week was almost the same in each celecoxib group, it is considered that both apoptosis induction in tumor cells and anti-angiogenesis in tumor stroma by celecoxib administration were at least attributable to prolonged survival of DMBA-induced carcinoma-bearing hamsters. Another factors influencing prolonged survival are now under investigation.

Several recent studies have demonstrated unequivocally that certain NSAIDs cause anti-inflammatory and antiproliferative effects independent of COX activity and PG synthesis inhibition (46). Niederberger et al. (47) showed that celecoxib, in contrast to other NSAIDs, did not inhibit but activate NF-κB and NF-κB–dependent gene transcription, suggesting that its COX-independent actions may differ from those of non-selective agents. In addition, celecoxib has been reported to inhibit cancer cell growth by arresting the cell cycle (48, 49). We recently reported up-regulation of p27 by celecoxib in some head and neck cancer cell lines, suggesting celecoxib induce cell cycle arrest (37).

In conclusion, our present results clearly indicate COX-2 protein to be increasingly expressed in the dysplastic pre-cancerous lesions and SCCs, and provide the evidence that celecoxib, a selective COX-2 inhibitor, possesses the chemopreventive potential against the DMBA-induced hamster cheek pouch carcinogenesis indicated by delayed onset of tumor development, retarded tumor growth and prolonged survival time. These effects were considered to be partly because of the anti-angiogenic action and apoptosis induction by celecoxib. From these findings, it was indicated that celecoxib could serve as a potent chemopreventive agent with low toxicity against oral carcinogenesis.

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