Chemoprevention by Nimesulide, a Selective Cyclooxygenase-2 Inhibitor, of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced Mammary Gland Carcinogenesis in Rats

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Breast cancer is common in women all over the world, and exploration of chemopreventive approaches to this cancer is very important. Nimesulide, a selective inhibitor of cyclooxygenase-2 (COX-2), is a good candidate as a chemopreventive agent with low toxicity. We examined its effects on mammary tumor development in female Sprague-Dawley rats induced with the environmental carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Rats at 7 weeks of age received intragastric intubations of PhIP (85 mg/kg body weight) 4 times weekly for 2 weeks and were maintained on control diet (high fat diet) or experimental diet (high fat diet supplemented with 400 ppm nimesulide) throughout the experiment. COX-2 protein was over-expressed in epithelial cancer cells and stromal cells of the PhIP-induced mammary carcinomas, but was weak or not apparent in normal mammary gland cells. The development of mammary carcinomas was clearly suppressed by administration of nimesulide. The carcinoma incidence was 51% as compared to 71% for the control diet group. The average multiplicity of carcinomas in the experimental diet group was 1.2±0.2 ($P<0.05$), significantly smaller than the control diet group value (2.6±0.5). The size of carcinomas was also clearly decreased; 1.1±0.4 cm³/rat in experimental diet group ($P<0.05$), 4.1±1.3 cm³/rat in the control diet group. The results therefore provide evidence that the selective COX-2 inhibitor, nimesulide, possesses chemopreventive activity against PhIP-induced mammary carcinogenesis in rats.

Key words: Nimesulide — COX-2 inhibitor — Chemoprevention — Mammary gland cancer — PhIP

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to decrease prostanoid synthesis through inhibition of cyclooxygenase (COX) activity,[1] resulting in anti-inflammatory effects. Two isoforms of cyclooxygenase-1 and -2 (COX-1 and COX-2) have been characterized in mammalian and avian species. COX-1 is constitutively expressed in most tissues to regulate prostaglandin (PG) production and maintain stable physiological conditions, including gastric cytoprotection and blood flow. In contrast, COX-2 is transiently induced by lipopolysaccharide, cytokines and growth factors, and has been indicated to produce large amount of prostanoids involved in inflammation and mitogenesis.[2] COX-2 protein and mRNA are known to be expressed in not only inflammatory tissues, but also colorectal cancers in rats and humans.[3, 4] Consistent with a causal role, regular use of aspirin has been shown to lower the risk of colon cancer in man.[5] Animal model studies have also demonstrated that NSAIDs, including aspirin and sulindac, can suppress colon carcinogenesis induced by azoxymethane (AOM) in rats.[6, 7] In

addition, NSAIDs have been shown to inhibit chemically induced mammary carcinogenesis in rats.[8, 9] Conventional NSAIDs such as aspirin, sulindac and indomethacin block both COX-1 and COX-2, resulting in unwanted side effects such as gastritis and gastric ulceration. However, selective COX-2 inhibitors have little gastric influence. Therefore, when NSAIDs are used over a long period as chemopreventive agents for mammary carcinogenesis, selective COX-2 inhibitors have an advantage over conventional NSAIDs. Nimesulide (4-nitro-2-phenoxymethanesulfonanilide), employed clinically in some European countries since 1985, has been reported as a selective COX-2 inhibitor.[10–12] In fact, the anti-inflammatory activity of nimesulide is almost the same as that of indomethacin, but its ulcerogenic potential is much weaker.[11]

There are controversial data regarding COX-2 expression and the effectiveness of NSAIDs in human breast cancer. COX-2 expression was found to be detected in 13 of 13 breast cancer samples by Parrett *et al.*[13] In contrast, it was reported that COX-2 protein was over-expressed only in 2 of 44 breast cancers and the level of COX-1 protein was increased in 30 of 44 cancers compared with normal mammary glands.[14] Epidemiological studies have

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shown a statistically significant reduction of breast cancer with the use of NSAIDs,[15, 16] although some findings have not been consistent with these data. Three prospective studies and one control study revealed no statistically significant relationship between the use of NSAIDs and the risk of mammary cancer development.[17–20] Thus, further investigations are required to determine the role of COX-2 in neoplastic development in the human breast.

In rodent mammary carcinogenesis models, there have been no reports as to whether selective COX-2 inhibitors show inhibitory effects on the development of mammary cancer. Moreover, the mechanism by which NSAIDs may inhibit mammary gland carcinogenesis is unclear. Therefore, in the present study, we investigated the chemopreventive efficacy of nimesulide on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a carcinogenic heterocyclic amine-induced mammary gland carcinogenesis in rats,[21, 22] and also the expression of COX-2 protein in the cancer.

MATERIALS AND METHODS

Chemicals and animals PhIP·HCl was purchased from the Nard Institute (Osaka). Nimesulide was kindly provided by Helsinn Healthcare-SA (Pazzallo-Lugano, Switzerland). Female Sprague-Dawley rats, purchased from CLEA Japan Inc. (Tokyo), were housed at $24\pm2°C$ and 55% humidity, with a 12-h light/dark cycle. The experimental diet was prepared by mixing 400 ppm nimesulide, a dosage which had been shown in previous studies to be effective for chemoprevention without any side effects,[23–25] with AIN-76A containing 23.5% corn oil (CLEA Japan Inc.) and stored in a cold room. Fresh diet was provided to rats once a week. During these processes, dietary nimesulide was confirmed to be stable.

Experimental procedure A total of 108 rats were divided into a PhIP-treated control diet group (42 rats, PhIP+high fat diet), PhIP-treated experimental diet group (42 rats, PhIP+high fat diet supplemented with nimesulide) and vehicle-treated groups (12 rats, water+high fat diet; 12 rats, water+high fat diet supplemented with nimesulide). At 7 weeks of age, all animals except those intended for vehicle treatment received intragastric intubations of PhIP (85 mg/kg body weight) 4 times weekly for 2 weeks, as described previously.[9] The experimental and control diets and water were given to animals *ad libitum* starting on the day of first administration of PhIP until the end of the experiment. Body weight and diet intake were measured weekly. Animals were monitored daily for their general health and mammary tumor development. The experiment was terminated 20 weeks after the first administration of PhIP because the palpable mammary tumor incidence in the control diet group had reached 55% at 18 weeks and the subsequent rate of incidence was small. All animals were killed under ether euthanasia and complete autopsies were performed.

At autopsy, the numbers and sizes of all mammary gland tumors were recorded. The length (*L*), width (*W*) and depth (*D*) of each lesion were measured with calipers and tumor volume (*V*) was calculated using the formula $V=L·W·D·\pi/6$. Half of each tumor was fixed with 10% formalin neutral buffer solution and the remaining tissue was frozen for subsequent immunoblot analysis. For histological examination, formalin-fixed tumors were embedded in paraffin and 5 μm sections were prepared and stained with hematoxylin and eosin. Pathological diagnosis of mammary gland tumors was made according to the criteria described previously.[26] Other organs showing apparent abnormalities were also embedded in paraffin, and sections were stained with hematoxylin and eosin for histological examination.

Immunoblot analysis Frozen mammary gland tumors were homogenized in RIPA buffer [50 mM tris-HCl buffer (pH 7.5) containing 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride and 10 μM leupepsin] and sonicated for 15 s. The sonicates were centrifuged and aliquots of the supernatant containing 30 μg protein were electrophoresed on 5–20% SDS/polyacrylamide gradient gels and transblotted onto polyvinylidene difluoride transfer membranes (Bio-Rad, Hercules, CA). Purified sheep seminal vesicle cyclooxygenase (Cayman, Ann Arbor, MI) and LPS+IFN-γ stimulated mouse macrophages lysate (Transduction Laboratories, Lexington, KY) were used as positive controls for COX-1 and COX-2, respectively, and prestained SDS-polyacrylamide gel electrophoresis (PAGE) standards (Bio-Rad) were employed as molecular weight markers. Blots were blocked with "Block Ace" (Dainippon Chemical, Tokyo) and incubated with mouse monoclonal anti-COX-2 antibody (Transduction Laboratories) or goat polyclonal anti-COX-1 antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA) at 1:1000 dilution at room temperature for 2 h. Then, the membranes were treated with horseradish peroxidase conjugated anti-mouse IgG (Amersham Int., Buckinghamshire, UK) at 1:6000 dilution or anti-goat IgG (Santa Cruz Biotech. Inc.) at 1:20 000 dilution for 1 h and developed with the enhanced chemiluminescence (ECL) western blotting system (Amersham Int.).

Immunohistochemical staining Formalin-fixed, paraffin-embedded tissue was processed as described above. Sections of tissue were cut into 4 μm thickness and mounted onto glass slides. The sections were deparaffinized and dehydrated with 0.3% hydrogen peroxide in methanol for 30 min and boiled in 10 mM citrate buffer (pH 6.8) for 15 min. The sections were blocked with 2% horse or rabbit serum and incubated with mouse monoclonal anti-COX-2 antibody or goat polyclonal anti-COX-1 antibody at 1:100

dilution overnight in a humidified chamber in a cold room. The same sections were incubated without the primary antibody as a negative control, and the sections from AOM-induced colon cancer in rats[27] were employed as a positive control. After washing of the sections, biotinylated horse anti-mouse IgG or rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA) was applied, and the sections were incubated at room temperature. The sections were washed and incubated in a horseradish peroxidase avidin-biotin complex reagent (Vector Laboratories) at room temperature for 30 min, then washed and incubated in 1 mg/ml 3,3′-diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M tris-HCl buffer (pH 7.6). Following this, the sections were counterstained with hematoxylin.

Statistical analysis Body weights, cancer incidences, cancer multiplicities and cancer volumes per rat were compared between the PhIP- and vehicle-treatment groups. Data for body weights, multiplicity and cancer volume per rat were analyzed with Welch's t test and for incidence by the χ^2 test. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Dietary administration of nimesulide at a dose of 400 ppm did not affect the diet intake of the PhIP-treated and vehicle-treated rats. Fig. 1 shows the change of body weight in the PhIP-treated and vehicle-treated groups. The average body weights of rats fed the control and experimental diets in the PhIP-treated groups were comparable throughout the experiment. The average daily intake of nimesulide in rats of the PhIP-treated group was calculated to be approximately 35 mg/kg body weight. During the experiment, 5 rats in the PhIP+experimental diet group died of acute pneumonia during PhIP intubations, and these were not included in the effective numbers.

At 12 weeks after the first administration of PhIP, palpable mammary gland tumors were already detectable in rats of the control diet group. Fig. 2 shows the time course of change in palpable mammary tumor incidence and multiplicity. Both incidence and multiplicity values for rats fed the experimental diet containing nimesulide were less than those in rats fed the control diet. Incidences and multiplicities of palpable tumors were 57% and 1.0±0.2/rat for the control diet group, and 30% and 0.6±0.2/rat for the experimental diet group, respectively, at experimental week 20. Additional non-palpable small tumors were also detected at the termination.

Histological examination revealed that all mammary gland tumors in both groups were invasive ductal carcino-
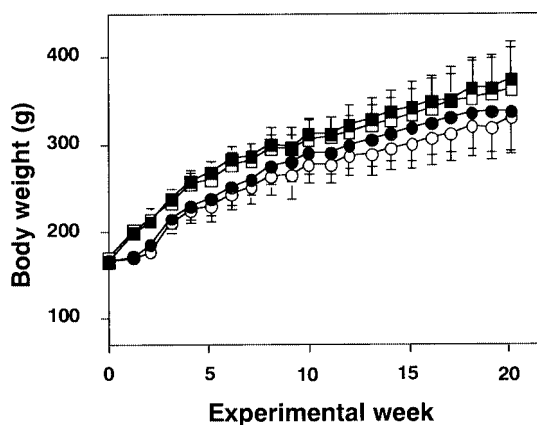

Fig. 1. Effects of PhIP and nimesulide on the body weight of rats. Closed circle, PhIP+control diet; open circle, PhIP+ experimental diet (400 ppm nimesulide); closed square, vehicle+ control diet; open square, vehicle+experimental diet (400 ppm nimesulide). Data are mean±SD values.
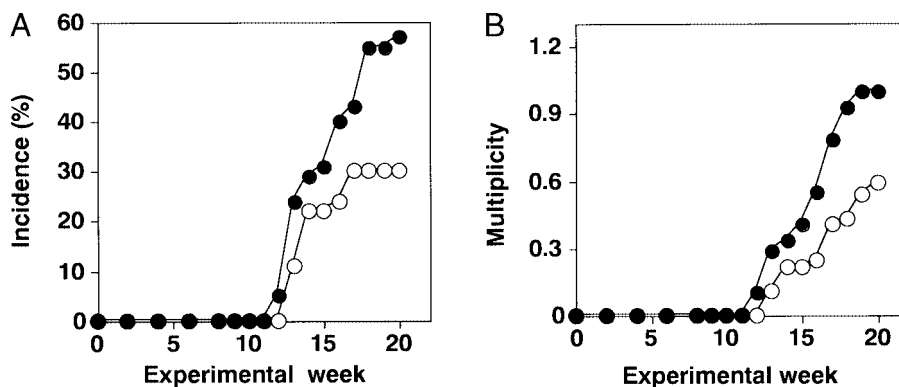

Fig. 2. Time course of palpable mammary tumor development in rats treated with PhIP. Panel A shows data for incidence and the panel B for multiplicity (average number of tumors per rat). Closed circles, PhIP+control diet; open circles, PhIP+experimental diet.

mas with one exception. The exception was an intraductal carcinoma. Table I summarizes the data of final mammary cancer incidences, multiplicities and cancer volumes per rat. The incidence of mammary cancer was 71% for the control group and 51% for the experimental group. Multiplicity and the volume of carcinomas were significantly decreased by nimesulide: 2.6 ± 0.5 in the control diet group and 1.2 ± 0.2 in the experimental diet group. Moreover, the volumes of carcinomas were 4.1 ± 1.3 cm³/rat and 1.1 ± 0.4 cm³/rat, respectively. No neoplastic lesions, other than those in the mammary gland, were found in the PhIP-treated control and experimental diet groups. Furthermore, in the vehicle-treated group animals, chronic administra-

tion of 400 ppm nimesulide did not produce any gross or histological changes in any organs, including liver, kidney, stomach, intestine and lung.

We confirmed the expression of COX-2 protein in PhIP-induced mammary carcinomas of various sizes (0.02 to 9.6 cm³) in the control diet group using an immunoblot analysis. All eight mammary tumors, diagnosed as invasive ductal carcinomas, showed COX-2 protein expression, with no apparent dependence on the dimensions. Analysis of COX-2 protein expression in eight carcinoma samples from the experimental diet group revealed similar expression levels as in the control diet group (Fig. 3). In addition, COX-1 protein expression was detected in all eight

Table I. Effects of Nimesulide on the Incidence, Multiplicity and Volume of Mammary Carcinomas Induced by PhIP in Female Sprague-Dawley Rats

	Effective No. of rats	Incidence (No. of rats with cancers)	Multiplicity[a] (No. of cancers /rat)	Cancer volume/rat[a] (cm³)
PhIP-treated				
Control diet	42	30/42 (71)[b]	2.6 ± 0.5	4.1 ± 1.3
Experimental diet (400 ppm nimesulide)	37	19/37 (51)	1.2 ± 0.2[c]	1.1 ± 0.4[c]

a) Data are mean±SE values.
b) Numbers in parentheses are percentage values.
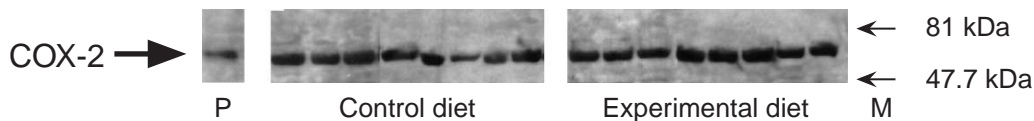c) Significantly different from the control diet group by Welch's t test ($P<0.05$).

Fig. 3. Immunoblot analysis for COX-2 in carcinomas selected from separate animals in the PhIP+control diet group and PhIP+experimental diet group. Aliquots of cellular lysate were electrophoresed and transferred to membrane filters, and COX-2 protein was detected using a COX-2-specific antibody. P and M indicate positive control for COX-2 and molecular weight markers, respectively.
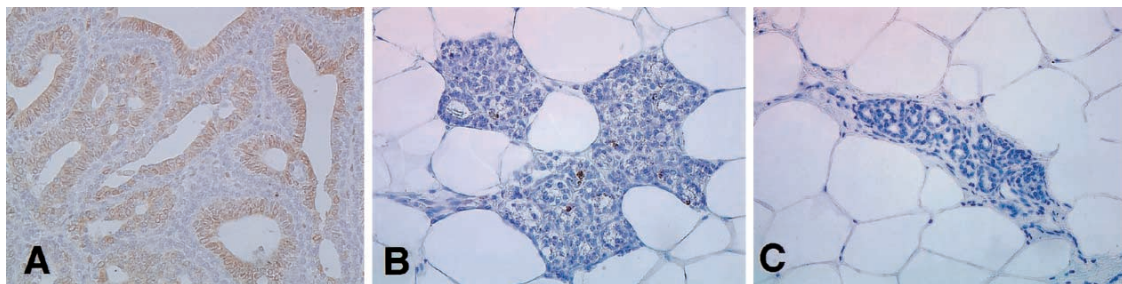

Fig. 4. Immunohistochemical staining for COX-2. Mammary carcinomas (A) and apparently normal mammary glands (B) from rats treated with PhIP+control diet, and normal mammary glands (C) from a rat treated with vehicle+control diet were examined. ×20 original magnification.

carcinomas from the control diet group, as well as eight carcinoma samples from the experimental diet group (data not shown).

Immunohistochemical analysis was performed to determine which cells expressed COX-2 in mammary gland carcinomas and whether normal mammary glands of PhIP-treated or vehicle-treated rats expressed COX-2 or not. In all mammary gland carcinomas tested in experimental and control diet groups, positive staining for COX-2 was localized in both epithelial cancer cells and stromal cells (Fig. 4A). In contrast, apparently normal mammary gland tissues from PhIP-treated rats infrequently expressed immunoreactive COX-2 in epithelial cells (Fig. 4B). Moreover, almost no COX-2-positive cells were detected in normal mammary glands from vehicle-treated rats (Fig. 4C). On the other hand, positive staining for COX-1 was detected in epithelial and stromal cells in mammary gland carcinomas and apparently normal mammary gland tissues from PhIP-treated rats and normal mammary gland tissues from vehicle-treated rats (data not shown).

DISCUSSION

In the present study, we demonstrated a clear chemopreventive efficacy of the selective COX-2 inhibitor, nimesulide, against PhIP-induced mammary gland carcinogenesis in rats. In addition, nimesulide did not cause any toxic lesions or side effects such as gastrointestinal bleeding in this experiment. Our previous studies indicated that nimesulide suppresses aberrant crypt foci formation in the colon of F344 rats treated with AOM,[28] development of intestinal polyps in Min mice[23] and colon carcinogenesis induced by AOM in ICR mice.[24] Okajima et al. have further demonstrated that nimesulide reduces the development of rat urinary bladder carcinomas caused by N-butyl-N-(4-hydroxybutyl)nitrosamine.[25] Recently, other selective COX-2 inhibitors, such as celecoxib, NS-398 and MF-tricyclic, have also been found to exert chemopreventive potential against small and large intestinal carcinogenesis in rats and mice.[29–31] The accumulating results suggest that selective COX-2 inhibitors are good candidates as chemopreventive agents for cancers, such as in the colon and breast. Some effort to assess the efficacy of nimesulide as a chemopreventive agent compared to other potential chemopreventive compounds, including conventional NSAIDs and other selective COX-2 inhibitors for breast cancer, in experimental animal models seems worthwhile.

The present study demonstrated that all the PhIP-induced mammary tumors tested, of various sizes, uniformly expressed COX-2 protein. Immunohistochemical studies showed that immunoreactive COX-2 was principally observed in mammary carcinoma tissues and also sometimes in apparently normal mammary glands in PhIP-treated rats. However, COX-2-positive cells were not able to be detected in normal mammary glands in vehicle-treated rats. These data indicate that COX-2 expression was increased in PhIP-induced carcinomas compared with normal mammary glands. As demonstrated in the present study, a selective COX-2 inhibitor, nimesulide, suppressed PhIP-induced mammary gland carcinogenesis, indicating that COX-2 plays a pivotal role in PhIP-induced mammary carcinogenesis in rats.

It has been reported that NSAIDs, including nimesulide, inhibit COX-2 catalytic activity, but do not affect the expression level of COX-2 in vitro or in vivo.[11, 32, 33] Consistent with these data, immunoblot and immunohistochemical analysis in the present study indicated that nimesulide did not alter the level or localization of COX-2 expression. On the other hand, dexamethasone, a glucocorticoid anti-inflammatory drug, is known to inhibit PG synthesis by blocking expression of COX-2.[2, 34, 35] Overexpression of COX-2 in colon cancer cell lines has been reported to result in resistance to apoptosis and an increase in metastasis and angiogenesis.[33, 36] To clarify the mechanisms of the chemopreventive effects of nimesulide, it is important to estimate differences of cell proliferation in mammary gland carcinomas of rats with or without nimesulide treatment. Moreover, the effect of nimesulide administration on PhIP metabolic activation should be examined. On the other hand, it has not yet been clarified whether the tumor-suppressive effects of NSAIDs are related to a reduction in prostaglandin or other independent mechanisms. It is very important to examine which types of prostanoids and receptors are required for the development of cancer in the breast.

In conclusion, administration of nimesulide, a selective COX-2 inhibitor, suppressed the development of PhIP-induced mammary cancers, in which COX-2 protein was over-expressed, in female Sprague-Dawley rats. These results suggest that nimesulide may serve as an effective chemopreventive agent, with low toxicity, against mammary gland cancer development in man.

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