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A CYCLOOXYGENASE-2 INHIBITOR, NIMESULIDE, INHIBITS POSTINITIATION PHASE OF *N*-NITROSOBIS(2-OXOPROPYL)AMINE-INDUCED PANCREATIC CARCINOGENESIS IN HAMSTERS

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The modification effects of nimesulide, a cyclooxygenase (COX)-2 inhibitor, administration during the postinitiation phase of pancreatic carcinogenesis were investigated in hamsters treated with *N*-nitrosobis(2-oxopropyl)amine (BOP). Male Syrian hamsters were given 4 weekly s.c. injections of BOP at a dose of 10 mg/kg and thereafter administered 0, 100 or 400 ppm nimesulide in the diet for 36 weeks. Additional groups of hamsters were fed 400 ppm nimesulide without prior BOP initiation or nontreated. At week 40, all surviving animals were killed and development of neoplastic and preneoplastic lesions was assessed histopathologically. The incidence of pancreatic adenocarcinomas was significantly ($p < 0.05$) decreased in the BOP/400 ppm nimesulide group compared to the BOP alone group. The multiplicity of total lesions of pancreatic adenocarcinoma plus atypical hyperplasia was also significantly ($p < 0.05$) lowered. Immunohistochemically, COX-2 was clearly expressed in pancreatic and lung tumor cells, whereas expression was not remarkably affected by the 400 ppm nimesulide treatment. Proliferating cell nuclear antigen labeling indices of pancreatic ducts were significantly ($p < 0.01$) reduced by nimesulide. The incidence and multiplicity of neoplastic lesions in other organs did not significantly differ among the BOP-treated groups, though only the multiplicity of lung tumors showed a tendency to decrease. No neoplastic lesions were detected in animals receiving nimesulide alone. Our results clearly indicate that nimesulide protects against BOP-induced pancreatic tumors in hamsters.

Key words: *nimesulide; pancreatic carcinogenesis; hamster;* N-*nitrosobis(2-oxopropyl)amine*

Pancreatic cancer is one of the cancers steadily increasing in incidence in Japan, with 5-year survival rates being <25%.[1,2] Although a number of approaches have been used to treat pancreatic cancers, median survival after diagnosis is only 19 months.[2] Likewise, development of pancreatic cancers is clinically so silent in general that at the time of diagnosis the vast majority of cases have been incurable with a very poor prognosis. Therefore, effective new approaches against this aggressive disease are urgently required.

Cyclooxygenase (COX) is an important enzyme involved in the arachidonate cascade, and 2 isoforms, COX-1 and COX-2, have been identified.[3,4] COX-1 is constitutively expressed and associated with the production of prostaglandins regulating vascular homeostasis, physiologic functions of the stomach and reabsorption of sodium and water in the kidney.[3,4] However, COX-2 is induced in association with inflammation and in a variety of cell proliferative conditions.[3,4] COX-2 has been closely associated with carcinogenesis, especially the growth and progression of a number of human cancers, including those in the colon,[5] stomach,[6] liver,[7] lung[8] and pancreas.[9,10] In addition, selective COX-2 inhibitors prevent growth of cancer cells.[4] It is now clear that COX-2 is an inducible immediate early gene,[11] involved not only in inflammation and cell proliferation[11] but also in differentiation,[12] apoptosis,[13] metastasis,[14] immunologic surveillance[15] and angiogenesis.[16]

Nimesulide, a preferential COX-2 inhibitor of nonsteroidal antiinflammatory drugs (NSAIDs),[17] inhibits chemically induced colon,[18,19] mammary,[20] tongue[21] and urinary bladder[22] carcinogenesis in rats. However, it is still important to examine the modifying effects of nimesulide on pancreatic carcinogenesis because the postinitiation and promotion stages are supposed to be organ-specific. Previously, we also reported that NSAIDs such as indomethacin and phenylbutazone can reduce the development of pancreatic cancer when administered during the postinitiation phase in the hamster *N*-nitrosobis(2-oxopropyl)amine (BOP) model,[23] strongly supporting the present study with nimesulide.

BOP induces pancreatic, lung, liver and kidney tumors in hamsters.[24] This model is considered to have particular advantage for assessing modification effects of chemicals on pancreatic carcinogenicity because of the histologic and biologic similarities of the induced lesions to those observed in humans.[24] The present experiment was performed to elucidate the effects of nimesulide during the postinitiation phase in the BOP-induced carcinogenesis hamster model.

MATERIAL AND METHODS

Animals and chemicals

A total of 110 male Syrian hamsters (Japan SLC, Shizuoka, Japan), 5 weeks old and weighing about 70 g, were used in this experiment. Animals were housed 5/polycarbonate cage in an air-conditioned room at 23 ± 2°C and 60 ± 5% humidity under a daily 12 hr light/dark cycle. A standard basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water were available *ad libitum.* BOP was obtained from Nacalai Tesque (Kyoto, Japan), and nimesulide was kindly provided by Helsinn Healthcare (Pazallo-Lugano, Switzerland).

Experimental protocol

As shown in Figure 1, groups 1–3, each consisting of 30 hamsters, were given BOP s.c. once a week for 4 weeks at a dose of 10 mg/kg body weight. During this postinitiation treatment, animals were continuously fed diet supplemented with 0 ppm

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(group 1), 100 ppm (group 2) or 400 ppm (group 3) nimesulide for 36 weeks. Doses were selected on the basis of previous experimental results for mice or rats.[19,20,22] Groups 4 and 5, consisting of 10 animals each, were fed a diet supplemented with nimesulide 400 or 0 ppm, respectively, without prior initiation. Hamsters were observed daily and weighed once every 4 weeks. Food consumption was measured weekly. At the end of week 40, all surviving animals were killed and examined histopathologically. Moribund or dead animals were also completely autopsied for histologic examination.

Histologic examination

At autopsy, the pancreas, stomach, lung, liver and kidney were carefully examined macroscopically, removed and fixed in 10% phosphate-buffered formalin. Before fixation, the latter 3 organs were weighed. As routinely processed, paraffin-embedded tissue sections, 3 μm thick, were stained with hematoxylin and eosin. To detect pancreatic proliferative lesions, 4 parts of the pancreas (splenic, duodenal and gastric lobes and head portion) were serially sectioned according to our routine method.[23,25–27] Neoplastic and preneoplastic lesions were histopathologically diagnosed as adenocarcinomas and atypical hyperplasias and counted in representative sections, as usually performed in our laboratory.[23,25–27] For COX-2 immunohistochemistry, the primary COX-2 polyclonal antibody (Cayman Chemical, Ann Arbor, MI) was used at a 1:100 dilution together with the Vectastain Elite ABC kit (Vector, Burlingame, CA) according to the modified method previously reported.[28] To examine mechanistic insights, cell proliferation was assayed by immunohistochemistry of proliferating cell nuclear antigen (PCNA),[29] and single-cell necrosis was assessed histologically. For visualization of immunohistochemical binding, diaminobenzidine was used.

Statistical evaluation

Quantitative results were statistically evaluated by ANOVA and Fisher's exact probability test.

RESULTS

Mean daily and total intakes of nimesulide were clearly related to the dietary dose levels (Table I). Survival rates and mean survival times in the BOP-treated groups (groups 1–3) were 63.3% and 253 days, 76.7% and 271 days and 83.3% and 269 days, respectively (Table I), showing that survival rates and mean survival times were somewhat greater in the nimesulide-treated groups than in the BOP alone group. Because the first moribund hamster in the BOP alone group (group 1) had pancreatic tumor at week 25 of the experiment, every animal was included in the effective number. Final body and relative organ weights were not significantly different among groups 1–3 (Table II).

Preneoplastic pancreatic lesions occurred in the splenic and gastric lobes and head portion without any clear skewing in lobe distribution by treatment (data not shown) and were histopathologically diagnosed as atypical hyperplasias, consisting of proliferation of atypical ductules accompanied by inflammatory cell infiltration, predominantly lymphocytic in character.[23,25–27] Pancreatic adenocarcinomas were well to moderately differentiated, showing distinct glandular patterns with severely atypical columnar or cuboidal epithelia.[23,25–27] Incidence and multiplicity data for histopathologically diagnosed pancreatic lesions observed in each group of hamsters are summarized in Table III. The incidence of pancreatic adenocarcinomas was significantly ($p < 0.05$) lower in group 3 than in group 1. Based on the incidence data for adenocarcinomas, 70% and 40% in groups 1 and 3, respectively, the

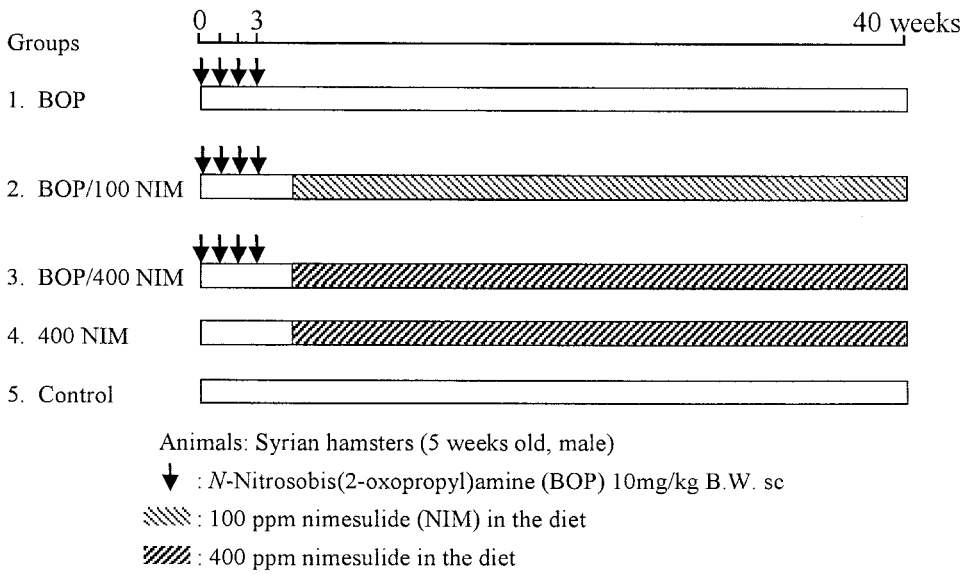

FIGURE 1 – Experimental design.

TABLE I – DATA FOR SURVIVAL, FOOD CONSUMPTION AND INTAKE OF NIMESULIDE

Group	Survival rate (%)	Mean survival (days)	Daily intake (g/hamster)	Total intake (g/hamster)
1. BOP	19/30 (63)	253 ± 42[1]	0	0
2. BOP/100 ppm nimesulide	23/30 (77)	271 ± 18*	0.07	18.2
3. BOP/400 ppm nimesulide	25/30 (83)	269 ± 26	0.27	70.2
4. 400 ppm nimesulide	10/10 (100)	280 ± 00	0.28	72.1
5. Control	9/10 (90)	262 ± 56	0	0

[1]Mean ± SD.–*$p < 0.05$ compared to group 1.

TABLE II – FINAL BODY AND RELATIVE ORGAN WEIGHTS

Group	Number of animals	Final body weight (g)	Lung (g%)		Liver (g%)	Kidney (g%)	
			Right	Left		Right	Left
1. BOP	19	180.6 ± 19.8[1]	0.43 ± 0.08	0.24 ± 0.09	6.10 ± 0.68	0.60 ± 1.01	0.35 ± 0.07
2. BOP/100 ppm nimesulide	23	178.8 ± 13.9	0.39 ± 0.05	0.24 ± 0.15	5.70 ± 0.72	0.37 ± 0.03	0.38 ± 0.04
3. BOP/400 ppm nimesulide	25	181.4 ± 13.7	0.44 ± 0.09	0.23 ± 0.05	5.97 ± 0.74	0.34 ± 0.02	0.36 ± 0.03
4. 400 ppm nimesulide	10	194.7 ± 17.3	0.39 ± 0.10	0.20 ± 0.04	5.54 ± 0.20	0.36 ± 0.05	0.38 ± 0.04
5. Control	9	196.8 ± 8.8	0.35 ± 0.05	0.18 ± 0.02	5.07 ± 0.37	0.34 ± 0.02	0.35 ± 0.02

[1]Mean ± SD

TABLE III – INCIDENCE AND MULTIPLICITY DATA FOR PANCREATIC ADENOCARCINOMAS AND ATYPICAL HYPERPLASIAS

Group	Effective number of animals	Number of animals with (%)			Number of tumors/animal (mean ± SD)		
		ADC[1]	AH	Total	ADC	AH	Total
1. BOP	30	21 (70)	17 (57)	26 (87)	0.93 ± 0.79	0.87 ± 1.04	1.80 ± 1.35
2. BOP/100 ppm nimesulide	30	22 (73)	11 (37)	23 (77)	1.00 ± 0.83	0.43 ± 0.63	1.43 ± 1.04
3. BOP/400 ppm nimesulide	30	12 (40)*	12 (40)	20 (67)	0.57 ± 0.77*	0.47 ± 0.63	1.03 ± 1.03*

[1]ADC, adenocarcinoma; AH, atypical hyperplasia.–*$p<0.05$ compared to group 1.

inhibition rate by 400 ppm nimesulide was calculated to be 43%. The incidence of pancreatic atypical hyperplasias also showed a tendency to decrease in groups 2 and 3 compared to group 1, though this was not statistically significant. The multiplicity of pancreatic adenocarcinomas or total lesions of adenocarcinomas plus atypical hyperplasias was significantly ($p < 0.05$) lower in group 3 than in group 1.

Immunohistochemically, intact pancreatic islets and inflammatory cells showed appreciable amounts of COX-2. Therefore, only tissue sections in which islets were positively stained were selected for evaluation. COX-2 was clearly expressed in the cytoplasm of pancreatic adenocarcinoma cells and atypical hyperplastic cells, no binding being evident in epithelial cells of intact pancreatic ducts or acini. In comparison to the staining intensity for islet cells, atypical hyperplastic and adenocarcinoma cells were positive for COX-2 similarly to or more strongly than islets, expression of which was comparable between groups 1 and 3 (Table IV). To assure immunohistochemical stainability, the same tissue sections as those positive for COX-2 in the islets were evaluated for PCNA labeling. PCNA labeling indices of pancreatic adenocarcinomas were significantly ($p < 0.01$) reduced by nimesulide (Table IV), though those of atypical hyperplasias showed only a tendency to decrease. Single-cell necrosis or apoptosis in pancreatic adenocarcinomas and atypical hyperplasias was not clearly affected (data not shown).

As summarized in Table V, neoplastic lesions were also observed in the lungs, liver and kidneys of hamsters receiving BOP. However, the incidence and multiplicity of these tumors were not significantly influenced by the nimesulide treatment, though lung tumors showed a tendency to decrease. Immunohistochemically, COX-2 was clearly expressed in lung adenocarcinomas and adenomas in a lesion severity-dependent manner but not in liver and kidney tumors, though again nimesulide treatment did not affect COX-2 expression in lung tumor cells (data not shown). No neoplastic lesions were noted in groups 4 and 5. There was no macroscopic evidence of bleeding or defects on the stomach mucosa in any group.

DISCUSSION

Our results provide strong evidence of the chemopreventive effects of nimesulide administration during the postinitiation phase of pancreatic carcinogenesis in hamsters treated with BOP, which is in good agreement with recent epidemiologic data.[30] Under the applied experimental conditions, nimesulide did not cause any adverse effects, such as body or organ weight changes or tumor-promotion activity, in any organ. Our previous studies indicated that the NSAIDs indomethacin and phenylbutazone could also suppress pancreatic carcinogenesis of hamsters treated with BOP without any adverse effects.[23] Because the inhibition rates by indomethacin and phenylbutazone for pancreatic adenocarcinomas were, respectively, 48% and 26%, the value at 43% for nimesulide found in the present study showed as efficacious protection as indomethacin.

Enhancement of COX-2 expression in human tumor cells has been reported for various cancers, including those arising in the colon,[5] stomach,[6] liver,[7] lung[8] and pancreatic duct,[9,10] thus suggesting that upregulation of this enzyme might be a common characteristic of epithelial neoplasms. In the present study, COX-2 was clearly expressed in hamster pancreatic adenocarcinomas. Inhibition of tumors by NSAIDs is considered to involve the common property of COX suppression and the resultant reduction in levels of prostaglandins in tissues,[19] though prostaglandin levels were not analyzed in the present study. Evidence has suggested that COX-2 may be the prime target of NSAIDs for their chemopreventive effects. However, COX-2 inhibitors, including nimesulide, suppress the catalytic activity, but not the expression level, of COX-2 *in vitro* and *in vivo*.[16,31] Likewise, chemopreventive activity on carcinogenesis in a variety of organs has been reported for a number of other selective COX-2 inhibitors. For example, 3-(3,4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(*5H*)-furanone (MF tricyclic) reduced the number and size of intestinal polyps in $APC^{\Delta 716}$ knockout mice,[32] and celecoxib suppressed azoxymethane-induced colon carcinogenesis in rats.[33] In terms of the mechanisms underlying the chemopreventive effects of nimesulide, inhibition of cell proliferation in the target cells was observed in the present study, as reported for the colon.[34]

In the present study, COX-2 expression was also demonstrable in intact pancreatic islets and pancreatic hepatocytes but not in epithelial cells of the duct or acinus. COX-2 expression in the islets is in line with reports on human islets,[9,35] so islet cells may serve well as inner controls for COX-2 expression. Experiments using COX-2 inhibitors may allow a better understanding of the possible role of COX-2 in pancreatic islet physiology and the pathogenesis of diabetes mellitus because prostaglandin E_2 negatively regulates pancreatic islet function, *i.e.,* glucose-induced insulin secretion.[9]

Fukutake *et al.*[19] reported that nimesulide exerts a suppressive effect on azoxymethane-induced colon carcinogenesis in ICR mice without significant influence on liver and lung tumor development, which is in line with our data in the present study. Organ speci-

TABLE IV – COX-2 EXPRESSION AND PCNA LABELING INDEX (%)

Group	Number of lesions	COX-2 (%)				PCNA (Mean ± SD)	
		AH[1]		ADC		AH	ADC
		+	++	+	++		
1. BOP alone	22	7 (32)	0	6 (27)	9 (41)	23.0 ± 7.7	35.6 ± 8.3
3. BOP/400 ppm nimesulide	13	5 (38)	0	4 (31)	4 (31)	17.2 ± 6.6	24.4 ± 6.7*

[1]AH, atypical hyperplasia; ADC, adenocarcinoma. +, similarly positive to islets; ++, more strongly positive than islets.–*$p < 0.01$ *vs.* group 1.

TABLE V – SITES AND TYPES OF NEOPLASTIC LESION IN OTHER ORGANS

Organ	1. BOP (n = 30)		2. BOP/100 ppm nimesulide (n = 30)		3. BOP/400 ppm nimesulide (n = 30)	
	Incidence (%)	Number of lesions/animal	Incidence (%)	Number of lesions/animal	Incidence (%)	Number of lesions/animal
Lung						
Adenoma	28 (93)	4.20 ± 3.11[1]	28 (93)	3.93 ± 2.65	28 (93)	3.30 ± 2.45
Adenocarcinoma	12 (40)	0.60 ± 0.89	11 (37)	0.43 ± 0.63	11 (37)	0.47 ± 0.68
Liver						
Hepatocellular adenoma	9 (30)	0.37 ± 0.61	8 (27)	0.33 ± 0.60	9 (30)	0.37 ± 0.61
Hepatocellular carcinoma	3 (10)	0.10 ± 0.30	7 (23)	0.30 ± 0.65	3 (10)	0.10 ± 0.31
Cholangiocellular adenoma	2 (7)	0.07 ± 0.25	7 (23)	0.23 ± 0.43	3 (10)	0.10 ± 0.31
Cholangiocellular carcinoma	5 (17)	0.17 ± 0.38	5 (17)	0.17 ± 0.38	3 (10)	0.13 ± 0.43
Kidney						
Adenoma	2 (7)	0.07 ± 0.25	1 (3)	0.03 ± 0.18	0	
Adenocarcinoma	2 (7)	0.07 ± 0.25	0		1 (3)	0.07 ± 0.37
Nephroblastoma	1 (3)	0.03 ± 0.18	0		1 (3)	0.03 ± 0.18

[1]Mean ± SD.

ficity of NSAIDs was also encountered in a rat multiorgan carcinogenesis model, in which the chemopreventive effect of indomethacin was investigated,[36] lung carcinogenesis being inhibited but not hepatocarcinogenesis. The mechanisms underlying such variation in COX-2 inhibitor influence at the organ level remain to be clarified. In conclusion, in the present study, nimesulide proved remarkably efficacious at inhibiting pancreatic tumorigenesis in hamsters treated with BOP when given in the postinitiation phase.

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