Occurrence of mutations in the epidermal growth factor receptor gene in X-ray-induced rat lung tumors

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Epidermal growth factor receptor (EGFR) gene alterations have been found in human lung cancers. However, there is no information on the factors inducing EGFR mutations. In rodents, K-ras mutations are frequently found in many lung carcinogenesis models, but hitherto, Egfr mutations have not been reported. Their presence was therefore investigated in representative lung carcinogenesis models with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosobis(2-hydroxypropyl)amine (BHP), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and ethyl carbamate (urethane), as well as X-ray irradiation. With the chemical carcinogenesis models, no mutations were detected in Egfr, which is in clear contrast to the high rates observed in either codon 12 or 61 of K-ras (21/23 of the lung tumors induced with NNK, 4/5 with MeIQx, 1/4 with urethane and 7/18 with BHP). However, in the X-ray-induced lung tumors, Egfr mutations with amino acid substitution were observed in exons 18 and 21 (4/12, 33%), but no activating mutation of K-ras was detected. In addition, one and four silent mutations were identified in K-ras (exon 1) and Egfr (exons 18, 20 and 21), respectively. Most mutations in both Egfr and K-ras were G/C→A/T transitions (7/8, 88% and 31/34, 91%, respectively). Although, the mutational patterns in equivalent human lesions were not completely coincident, this first report of Egfr mutations in an experimental lung tumor model suggests that X-rays or other factors producing oxygen radicals could cause EGFR mutations in some proportion of lung cancers in humans. (Cancer Sci 2008; 99: 241–245)

Lung cancer is the major cause of death in both sexes in Japan and many parts of the world[1,2] so analysis of causative factors and development of preventive methods is important, in addition to advances in diagnostic and therapeutic methods. Genetic alterations (KRAS, TP53 etc.) in lung cancers have been studied in this context.[3]

Epidemiologic studies of lung cancers have pointed to many risk factors including tobacco smoking, air pollution, occupational environments, and ionizing radiations including radon.[4] Interestingly, dietary habits have also been demonstrated to influence the risk of lung cancer, with well-cooked red meat consumption demonstrated as a risk factor.[5,6] Tobacco smoking appears to be correlated with KRAS mutations.[3]

Recently, somatic mutations of the epidermal growth factor receptor (EGFR) gene, a tyrosine kinase of the ErbB family, have been reported to be frequent in human lung adenomas and adenocarcinomas, especially in Asians, women, and non-smokers.[7–10] However, factors inducing EGFR mutations are quite unclear. To prevent the presently increasing rates of lung adenocarcinomas, this question demands our urgent attention.

Experimental animal models of lung carcinogenesis have been established to elucidate mechanisms and to allow screening for enhancing and suppressing factors. Representative carcinogens inducing high incidences of lung cancers include: 4-(methylnit-rosamino)-1-(3-pyridyl)-1-butanone (NNK), found in tobacco smoke; N-nitrosobis(2-hydroxypropyl)amine (BHP), a synthesized carcinogen not existing in nature; 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), a heterocyclic amine having mutagenicity and carcinogenicity, which exists in cooked meat and fish;[5,6,11] ethyl carbamate (urethane) and X-rays. Mutations of K-ras have been reported in mouse and rat tumors, including NNK- and BHP-induced lung adenomas and adenocarcinomas.[12,13] However, alterations of Egfr have hitherto not been identified.

In the present study, to assess possible mutational factors impacting on Egfr, we investigated genetic alterations in Egfr exons 18–21, frequently found in human lung cancer[7–10] in a series of animal lung neoplasms induced by NNK, BHP, MeIQx, urethane and X-rays. For comparison, K-ras exons 1 and 2 were also analyzed.

Materials and Methods

Chemicals. NNK and urethane were purchased from Sigma (St Louis, MO, USA), MeIQx from Nard Institute (Nishinomiya, Japan), and BHP from Nakarai Tesuque (Kyoto, Japan).

Animal treatments. Experimental animals were purchased from Japan SLC, Inc. (Shizuoka, Japan) and each experimental treatment started after adaptation for a week.

To obtain animal lung tumor samples, animal experiments with five lung carcinogenesis models were carried out as follows.

For NNK-induced lung tumors, 7-week-old female A/J mice were given a single dose of NNK (2 mg/0.1 mL saline/mouse, i.p.), and then maintained without additional treatment until sacrificed at week 52. This experiment was conducted by M. Yokohira and K. Imaida.

For MeIQx-induced lung tumors, 7-week-old female A/J mice were given 600 p.p.m. MeIQx in a basal diet for 12 weeks, and were then maintained on the basal diet without MeIQx until sacrificed at week 32. This experiment was also conducted by M. Yokohira and K. Imaida.

For the urethane-induced lung tumors, 8-week-old female A/J mice were given a single dose of urethane (250 mg/kg, i.p.), and then maintained without additional treatment until sacrifice at week 50. This experiment was conducted by N. Takasuka.

The protocol for BHP-induced lung tumors was based on previous reports[14] and the experiment was conducted by M. Tsutsumi.

The protocol for X-ray-induced lung tumors was conducted by Y. Yamada and Y. Oghiso.[15] Briefly, for local thoracic X-irradiation, female Wistar (W/M) strain rats at the ages of 100–120 days were exposed to 3.0 Gy of X-rays and then maintained without additional treatment until sacrifice when moribund, dead, or at 24 months.

Table 1. Oligonucleotide primers for PCR amplification

Gene	Animal	Treatment	Exon	Primer sequence (5'-3')		Target codons[†]	Annealing temperature
K-ras	Mouse	MelQx, NNK	1	F; ACTGAGTATAAACTTGTGGT	R; CCTCTATCGTAGGGTCGTAC	9–30	53°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TTATGGCAAATACACAAAGA	50–77	53°C
		Urethane	1	F; AGGCCTGCTGAAAATGACTG	R; CCTCTATCGTAGGGTCGTAC	4–30	55°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TGGTGAATATCTTCAAATGATTTAGT	50–86	53°C
	Rat	BHP	1	F; AGGCCTGCTGAAAATGACTG	R; GCAGCATTTACCTCTATCGT	4–33	53°C
			2	F; AAGTAGTAATTGATGGAGAA	R; TGGTGAATATCTTCAAATGATTTAGT	50–86	53°C
		X-ray	1	F; TGACTGAGTATAAACTTGTGGTAGTTG	R; TCGTAGGATCATATCATTCCACAAG	11–26	57°C
			2	F; AAGTAGTAATTGATGGAGAA	R; GGCAAATACACAAAGAAAGC	50–76	53°C
Egfr	Mouse	MelQx, NNK	18	F; CTCCCTTCTTCACAGCTCG	R; TCTCCAGGATGTTACCTTATAC	692–727	55°C
			19	F; TTCTTAATCTCAGGGTCTCT	R; GAAAACTCACGTCAAGGAT	734–760	55°C
			20	F; GTCCTTACCTTGTAGGAAGC	R; TCCCAACGTGCTTACCTTTG	766–823	55°C
			21	F; GGGCATGAACTACCTGGAAG	R; AGGACTTACTTTGCCCCCCTC	833–873	55°C
		Urethane	18	F; CTCGTGGAACCTCTCACACC	R; ATGTTACCTTATACACTGTGCCAAATG	697–723	55°C
			19	F; CAAGTTAATGTCAGCCCTCTTC	R; TAAAAGAAAACTCACGTCAAGGATTTC	731–759	55°C
			20	F; AGGAAGCCTATGTGATGGCTA	R; GACGTAGTCCAGGAGGCAAC	771–797	60°C
			21	F; CCTCTGTATTTCAGGGCATG	R; ACTCCCAGGACTTACTTTGC	828–875	55°C
	Rat	BHP	18	F; TGGTGCTAGCATCTCTGGTC	R; AGTCCAGACCTGTCTCCAGG	689–729	55°C
			19	F; CAGGTTAATGTCAGCCCTCTTC	R; GGAAACCGTGGTTAGCAAGA	730–762	55°C
			20	F; CCCATCAGCCAAGAAACAAT	R; GTACTCCAGGGGGCAGACCT	763–824	55°C
			21	F; GGGCATGAACTACCTGGAAG	R; AGGACTTACTTTGCCCCCCTC	832–872	55°C
		X-ray	18	F1; TGGTGCTAGCATCTCTGGTC	R1; CTCCTGAACCCAGAACTTTGA	689–715	55°C
				F2; GGAGAAGCTCCGAACCAAG	R2; AGTCCAGACCTGTCTCCAGG	704–729	55°C
			19	F; CAGCCCTCTTCTTAATCTCAGG	R; GCAAGACATAAAAGGAAACTCACA	731–762	55°C
			20	F1; CACATGTGTTGTCCTTACCTTG	R1; AACCATAGGGCATGAGTTGTG	763–790	55°C
				F2; ACCTCCACTGTCCAGCTCAT	R2; GCAGACCTTCCAATGTGCTTA	791–824	55°C
			21	F1; TGAAGCGTCTTCTGTGTTTCA	R1; TTGGCCAGTCCAAAATCTGT	825–854	55°C
				F2; TACTGGTAAAGACACCACAGCA	R2; GCTTCCTGACTTATTCTCAGGACT	852–876	55°C

[†]Corresponding to mouse and rat codons. BHP, N-nitrosobis(2-hydroxypropyl)amine; Egrf, epidermal growth factor receptor; MelQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCR, polymerase chain reaction.

Table 2. Incidences of mutations with amino acid substitution and silent mutations of the K-ras and Egfr genes in animal lung neoplasms

Treatment	Animal	K-ras		Egfr			
		Exon 1	Exon 2	Exon 18	Exon 19	Exon 20	Exon 21
Mutations with amino acid substitution							
NNK	Mice	21/23 (91%)	0/22	0/23	0/22	0/15	0/14
MelQx	Mice	4/5 (80%)	0/5	0/5	0/4	0/4	0/5
Urethane	Mice	0/8	1/4 (25%)	0/4	0/4	0/5	0/3
BHP	Rats	7/18 (39%)	0/14	0/18	0/18	0/17	0/18
X-ray	Rats	0/12	0/11	3/12 (25%)	0/11	0/12	1/11 (9%)
Silent mutations							
X-ray	Rats	1/12 (8%)	0/11	1/12 (8%)	0/11	2/12 (17%)	1/11 (9%)

BHP, N-nitrosobis(2-hydroxypropyl)amine; Egrf, epidermal growth factor receptor; MelQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

All the studies were conducted according to the Guidelines for Animal Experiments in the respective facilities.

Tissue preparation and DNA extraction. Upon sacrifice, the lungs were immediately excised and portions were fixed in neutrally buffered 10% formalin and embedded in paraffin. Two serial thin sections were made, one of 3 μm thickness to be stained with hematoxylin and eosin for histological examination, and the other of 8 μm thickness for DNA extraction.

For analysis of K-ras and Egfr mutations, paraffin-embedded lung neoplastic lesions (alveolar hyperplasia [AH], adenoma [Ad] and adenocarcinoma [AC]) from each animal model were used. Neoplastic lesions were scraped off from paraffin sections using needles and DNA was extracted using DEXPAT (TaKaRa Shuzo, Shiga, Japan).

Polymerase chain reaction (PCR). K-ras and Egfr gene fragments were amplified by PCR from lung DNA samples. PCR primers were synthesized at Operon Biotechnologies Inc. (Tokyo, Japan) with oligonucleotide purification cartridge grade. The sequences and target codons are listed in Table 1. Different primer sets were used according to each sample quality. In some cases, one exon was analyzed with two short PCR products overlapping partially.

PCR for analysis of the gene alterations was performed in 50 μL of reaction mixture consisting of 0.5 μM of each primer, 10 × PCR buffer (Applied Biosystems, Foster City, CA, USA), 200 μM each dNTP, 2.5 U AmpliTaq Gold (Applied Biosystems) and 0.5–5 μL of template DNA. The mixture was heated at 94°C for 9 min and subjected to 50 cycles of denaturation (94°C, 30 s), annealing (at each temperature in Table 2, 30 s)

and extension (72°C, 1 min) using a thermal cycler, DNA Engine PTC-200 (Bio-Rad Laboratories Inc. Hercules, CA, USA).

Single strand conformation polymorphism (SSCP) analysis. SSCP analysis was conducted by the method of Orita *et al.* with modifications.[16] PCR products were treated using ExoSAP-IT (USB Corp., Cleveland, OH, USA) before application to SSCP analysis. Four and a half μL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol were added to 0.5 μL PCR products treated by ExoSAP-IT, heated at 90°C for 3 min, cooled at 4°C for 1 min and then applied to 5–20% gradient polyacrylamide gel (e-PAGEL, ATTO corporation, Tokyo, Japan). Electrophoresis was carried out at 300 V for 1.5 h at 4°C and the gels were soaked in 10% trichloroacetate and in 50% methanol for 10 min each. DNA bands were detected by silver staining using 2D Silver Staining Solution II (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan). Detected shifted bands were homogenated, heated and centrifuged with DEXPAT to extract DNA and again applied to PCR and direct sequencing for verification of the mutation.

Direct DNA sequencing. With 2 μL of the ExoSAP-IT-treated PCR products and 5′ or 3′ of each PCR primer (Table 1), cycle sequencing reactions were carried out using a DYEnamic ET terminator cycle sequencing kit (GE Healthcare UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, England) and the sequences were determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

Histological findings. Lung neoplastic lesions induced by each treatment mostly originated from alveolar type II cells or bronchiolar Clara cells. Almost all histopathological types of lung neoplasms with each treatment were epithelial types, being classified into AH, Ad, AC, adenosquamous carcinoma and squamous cell carcinoma categories. A total of 66 lesions, classified into 4 AHs, 7 Ads, and 12 ACs from NNK, 2 AHs and 3 Ads from MeIQx, 8 Ads from urethane, 15 Ads and 3 ACs from BHP, 4 Ads and 8 ACs from X-ray-treated animal lungs, respectively, were used in the present mutational analyses.

K-ras alterations in lung neoplastic lesions. Activating mutations of the K-*ras* gene at codons 12 and 61 were detected in neoplastic lesions induced by NNK (21/23; 91%), MeIQx (4/5; 80%), urethane

Table 3. Mutation patterns for the K-*ras* and *Egfr* genes in lung neoplasms

Treatment	Animal	Gene	Exon	Nucleotide alteration	Amino acid substitution[†]	Frequency
NNK	Mice	K-*ras*	1	GGT→GAT	G12D	21/21
MeIQx	Mice	K-*ras*	1	GGT→GAT	G12D	3/4
				GGT→GCT	G12A	1/4
Urethane	Mice	K-*ras*	2	CAA→CTA	Q61L	1/1
BHP	Rats	K-*ras*	1	GGT→GAT	G12D	7/7
X-ray	Rats	K-*ras*	1	GGA→GGT	G10	1/1
		Egfr	18	CCC→CTC	P695L	1/8
				GGA→GAA	G697E	1/8
				GTT→GTC	V718	1/8
				GGT→GAT	G720D	1/8
			20	ACC→ACT	T784	1/8
				CAG→CAA	Q788	1/8
			21	CAC→CAT	H836	1/8
				GGT→GAT	G864D	1/8

[†]Corresponding to mouse and rat codons. BHP, N-nitrosobis(2-hydroxypropyl)amine; *Egrf*, epidermal growth factor receptor; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

(1/4; 25%) and BHP (7/18; 39%), respectively, but not in X-ray-induced tumors (Table 2). Histological classifications in neoplasms with K-*ras* mutations were 3 AHs, 7 Ads and 11 ACs from NNK, 1 AH and 3 Ads from MeIQx, 1 Ad from urethane, 5 Ads and 2 ACs from BHP-induced neoplasms. Only one silent mutation was detected in a X-ray-induced Ad (1/12; 8% in Table 2). Mutations were mostly G/C→A/T transitions. In one neoplasm, each in the MeIQx, urethane and X-ray models, G/C→C/G (AH), A/T→T/A (Ad) and A/T→T/A (Ad) transversions, respectively, were observed (Table 3). Particular histological differences were not observed with each mutation pattern.

***Egfr* gene alterations in lung neoplastic lesions.** Lung neoplastic lesions induced by NNK, MeIQx, urethane and BHP were found to harbor activating K-*ras* mutations, but not *Egfr* mutations. On the other hand, *Egfr* mutations with amino acid substitution were detected in X-ray-induced tumors (4/12; 33% in Table 2). Representative mutation charts are shown in Fig. 1.
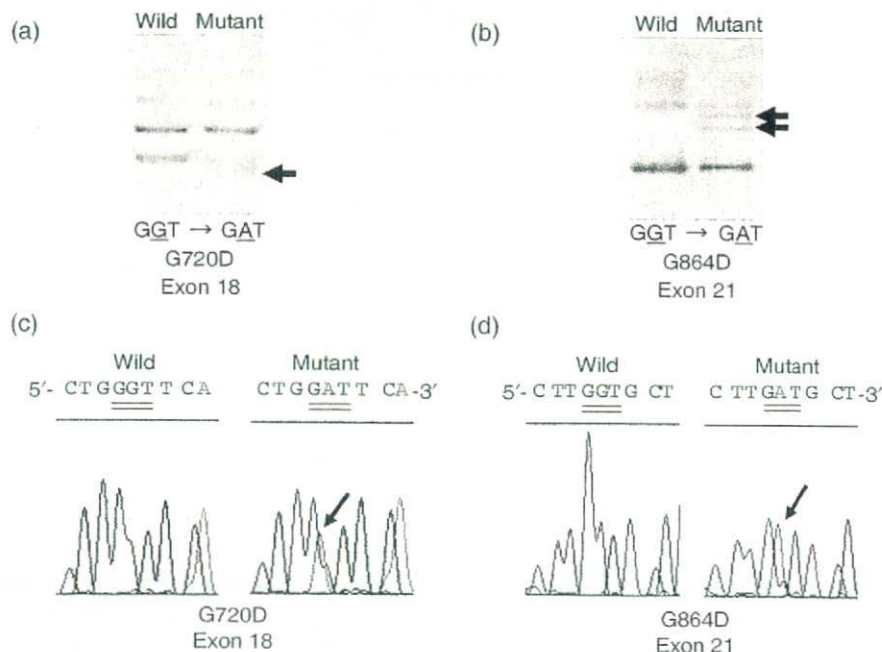

(a) Wild Mutant

GGT → GAT
G720D
Exon 18

(b) Wild Mutant

GGT → GAT
G864D
Exon 21

(c)

Wild
5′- CT GGG T T CA

Mutant
CT G GAT T CA -3′

G720D
Exon 18

(d)

Wild
5′- C TT GGTG CT

Mutant
C TT GAT G CT -3′

G864D
Exon 21

Fig. 1. Representative examples of single strand conformation polymorphism analysis (a, b) and charts of *Egfr* mutations confirmed by direct sequencing (c, d). Arrows in panels (a) and (b) indicate shifted bands associated with mutations. Panels a, c and b, d show G/C→A/T mutations found at codon 720 in exon 18 and at codon 864 in exon 21, respectively. Both arrows in panel (b) indicate the same mutation pattern of GGT to GAT at codon 864.

Fig. 2. Typical histological subtypes of X-ray-induced rat lung tumors with and without the *Egfr* mutations. (a) Papillary type adenocarcinoma. (b) Solid type adenocaricnoma. (c) Acinar type adenocarcinoma. The tumors of (a) and (b) exhibit the *Egfr* mutations and (c) is without the *Egfr* mutation. Histological classifications refer to our previous study.[15,20] Scale bars, 500 μm.

All the *Egfr* mutations were reconfirmed by sequencing of independent PCR products derived from the original template DNA. Mutation sites were mostly found in exon 18 (3/4) and the remainder in exon 21 (1/4). In addition to the mutations with amino acid substitution, silent mutations were also detected (4/12, 33%) in exons 18 (1/4), 20 (2/4) and 21 (1/4), respectively, two of the four silent mutations overlapping with mutations causing amino acid substitution. None of the tumors with *Egfr* mutations had K-*ras* mutations. *Egfr* alteration sites were not located in specific codons, but distributed over exons 18–21 (except 19) (Table 3). Tumors harboring *Egfr* mutations with amino acid substitution were histologically classified as 1 Ad and 3 ACs all with G/C→A/T patterns. Histological classifications of tumors with silent mutations of *Egfr* were 1 Ad with G/C→A/T, 3 ACs with 2 G/C→A/T and T/A→C/G patterns, respectively. Among these tumors, two ACs exhibited both missense and silent mutations, 1 AC with G/C→ A/T at codon 697 in exon 18 and G/C→A/T at codon 784 in exon 20, and 1 AC with G/C→A/T at codon 720 in exon 18 and T/A→C/G at codon 718 in exon 18, respectively. In addition, we further classified histological subtypes of the tumors with or without the *Egfr* mutations. Typical histological subtypes of X-ray-induced tumors used in the present study are shown in Fig. 2. Out of six tumors with the *Egfr* mutations, three were papillary type and the other three were solid type, while one papillary, one solid, three acinar and one bronchiolo-alveolar types were included in six tumors without the *Egfr* mutation. The papillary and solid types were frequent in the tumors with the *Egfr* mutations compared to the tumors without the *Egfr* mutation in the X-ray model.

Discussion

In the present study of mutation profiles of K-*ras* and *Egfr* in chemical and X-ray-induced lung carcinogenesis animal models, *Egfr* alterations were detected in 4 of 12 (33%) X-ray-induced tumors. To our knowledge, this is the first demonstration of mutations in *Egfr* in an animal lung carcinogenesis model. These mutations detected in this study are thought to be somatic, because experimental animals used for X-ray lung carcinogenesis were inbred and no mutations were detected in non-neoplastic parts of lung sections used for extracting DNA samples. The *Egfr* mutation sites and patterns found in X-ray-induced tumors have no codon specificity and were scattered over exons 18–21 (except 19). An amino acid substitution at codon 720 is reported as a mutation site in human lung cancer (corresponding to codon 719 in human EGFR).[10] This similarity suggests that the rat lung carcinogenesis model induced by X-rays may reflect, in part, human lung carcinogenesis with *EGFR* mutation. However, the other mutations differed from the most frequent mutations in human cases.

Yuan *et al.* reported that T/A→G/C substitutions, a change frequently detected in *EGFR* of human lung cancer, were induced by a 5.0 Gy dose of X-ray irradiation in mouse cells.[17] They speculated that this mismatch substitution resulted from repair activity of polymerase-β. However, in the present study, T/A→G/C substitutions were not detected in rat lung tumors induced *in vivo* by 3.0 Gy of X-ray irradiation. The differences between this study's data and Yuan's data could be due to differences in irradiation doses and species dependence regarding metabolism and repair systems.

In X-ray-induced tumors, silent mutations in *Egfr* and K-*ras* were also observed (Table 3). Generally, these have been believed to not affect events of protein levels, but a recent study demonstrated that silent mutations may also alter the conformation and activity of a protein.[18] Although half of the detected silent mutations (2/4) in *Egfr* overlapped with mutations generating amino acid substitution, some role in lung carcinogenesis could not be ruled out, at least in the other two cases.

In the histological evaluation, *Egfr* mutations were detected in both Ad and AC induced by X-rays in the lung, suggesting a possible involvement in an early stage of the neoplastic process, as with activating K-*ras* mutations. In the present study, the papillary type was frequently observed in the tumors with the *Egfr* mutations compared to the tumors without the *Egfr* mutations in the X-ray model. In humans, the papillary type was also predominant in lung cancers with *EGFR* mutations.[19] There are some similarities of the histological subtype between X-ray-induced lung tumors and human lung cancers with *EGFR* mutations.

In our previous study, immunohistochemical staining for surfactant apoprotein A and Clara cell 10 kDa protein have revealed that most of the lung tumors induced by X-rays originated from either type II alveolar or Clara cells.[20] On the bases of our previous data, we compared the expression of SP-A and CC-10 between the tumors with and without the EGFR mutations in the present study. However, no specificity of cell differentiation phenotypes was observed between tumors with and without the *Egfr* mutations.

In the present study, we conducted immunostaining for the Egfr downstream molecules of phospholyrated ERK (pERK) and Akt (pAkt). However, pERK and pAkt were mostly negative in both X-ray-induced tumors with and without the *Egfr* mutations (data not shown). No specificity of the expression patterns of pERK and pAkt were observed between tumors with and without the *Egfr* mutations. In the human lung cancer, Ikeda *et al.* reported that pAkt expression was significantly associated with the codon 858 mutation in the exon 21, but not in the exon 19 deletions, while pERK did not have any correlation.[21] The *Egfr* mutation patterns and sites detected in the present study were different from the above two mutations of human lung cancer. Moreover, to investigate the influence of the

Egfr mutations detected in the present study on cell proliferation activity, we also conducted immunostaining for proliferating cell nuclear antigen (PCNA). The ratio of PCNA positive cells tended to be higher in X-ray-induced lung tumors with the *Egfr* mutations ($22.1 \pm 6.9\%$ [mean $\pm$ standard deviation]) than those without the *Egfr* mutation ($13.9 \pm 3.7\%$). Further analysis such as transfection study in cell culture system is warranted to clarify the biological effect of the *Egfr* mutations, found in the present study.

The nitroso compounds, NNK and BHP, are well known to frequently induce K-*ras* mutations with G/C→A/T transitions in animal models[12,13] as confirmed in our present study. Lung tumors induced by MeIQx and urethane, non-smoking factors, were also found to harbor K-*ras*, but none of them featured any *Egfr* mutations. These data suggest that mutation hotspots might differ between chemical and X-ray mutagenesis.

Most chemical carcinogens modify DNA bases by forming adducts, whereas X-rays are known to cause genomic DNA damage, mostly having indirect effects, by producing oxygen radicals derived mainly from O_2 and H_2O molecules *in vivo*. These different mechanisms of DNA damages may contribute different gene targets. Indeed, the coexistence of both K-*ras* and *Egfr* mutations was not observed in the present study as observed in human cases. In addition to X-rays, microparticles, a factor of air pollutions derived from various industrial activities, might therefore also be likely to induce *EGFR* mutations because of their ability to produce oxygen radicals.[22]

Recently, the proportion of lung adenocarcinomas is increasing in our country. The prevalence of lowtar filter cigarettes makes smokers inhale more deeply and this is suggested to be a cause for recent increases. However, the reason for the increases of lung adenocarcinomas, especially with the *EGFR* mutations among non-smokers in Asia, is unclear. Ashakumary *et al.* reported that administration of a high-fat diet increases the concentration of lipid peroxides in rat lung tissue.[23] Recent changes of lifestyle may contribute to the increases of lung adenocarcinomas. The fact that endogenous oxygen radicals are produced by chronic inflammation is of interest in this context.[24] Clearly, the causative factors for *EGFR* mutations warrant further attention.

As an animal model for human lung adenocarcinomas, transgenic mice expressing mutant *EGFR* of human patterns in alveolar epithelium have been established.[25] That model is considered to be useful for developing therapeutic methods for human lung adenocarcinomas bearing *EGFR* mutations. The present model of X-ray-induced rat lung adenocarcinoma with the *Egfr* mutations may also be useful for studying lung carcinogenesis processes and developing therapeutic methods.

In conclusion, though the mechanisms of X-ray lung carcinogenesis have yet to be fully elucidated at the molecular level, in the present study we predominantly detected *Egfr* mutations in X-ray-induced lung tumors of rats. X-ray irradiation or endogenous factors producing oxygen radicals may thus cause *EGFR* mutations in some proportion of human lung cancers.

Acknowledgments

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Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice

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Adipocytokines are a group of adipocyte-secreted proteins that have significant effects on the metabolism of lipids and carbohydrates, as well as numerous other processes. A number of recent studies have indicated that some adipocytokines may significantly influence the proliferation of malignant cells *in vitro*, whereas it remains unclear whether they have similar roles *in vivo*. In this study, we determined serum levels of adipocytokines in mice with azoxymethane (AOM)- and dextran sulfate sodium (DSS)-induced colon carcinogenesis. Five-week-old ICR mice were given a single intraperitoneal injection of AOM followed by 1% DSS in drinking water for 7 days. Nobiletin (NOB), a citrus flavonoid, was given in the diet (100 p.p.m) for 17 weeks. Thereafter, the incidence and number of colon tumors and serum concentration of adipocytokines were determined at the end of week 20. The serum leptin level in AOM/DSS-treated mice was six times higher than that in untreated mice, whereas there were no significant differences in the levels of triglycerides, adiponectin and interleukin-6. Feeding with NOB abolished colonic malignancy and notably decreased the serum leptin level by 75%. Further, NOB suppressed the leptin-dependent, but not independent, proliferation of HT-29 colon cancer cells and decreased leptin secretion through inactivation of mitogen-activated protein kinase/extracellular signaling-regulated protein kinase, but not that of adiponectin in differentiated 3T3-L1 mouse adipocytes in a dose-dependent manner. Taken together, our results suggest that higher levels of leptin in serum promote colon carcinogenesis in mice, whereas NOB has chemopreventive effects against colon carcinogenesis, partly through regulation of leptin levels.

Introduction

Colorectal cancer was seen in about 1 million new cases throughout the world in 2002, with similar numbers for men and women. In terms of incidence, colorectal cancer ranks fourth in frequency in men and third in women (1). Epidemiological studies have provided abundant evidence that environmental factors, rather than genetic variations between populations, are of prime importance in the etiology of this disease (2,3). One of the most influential factors is obesity, whose prevalence has markedly increased over the past two decades, especially in industrialized countries (4). Obesity is known to increase the risk of several different chronic diseases, such as coronary heart disease, stroke and cancer (5,6). Further, the results of case–control and prospective studies suggest that obesity is a strong risk factor for

colorectal cancer, especially in men (7–10). More recently, a prospective population-based study of ~90 000 subjects conducted by the American Cancer Society confirmed that obesity is directly associated with an increased risk of death from colon cancer (11). Animal studies have confirmed that finding and also showed that obesity enhances tumor development (12), whereas calorie restriction inhibits a broad range of spontaneous, transplanted and chemically induced tumors (13). However, the mechanism underlying the development of obesity-associated colon cancer has not been fully elucidated.

Until the discovery of adipocytokines, adipose tissue was only thought to have passive functions as an energy storage depot and mechanical barrier. Adipocytokines are a group of adipose tissue-secreted hormones that were initially reported in the early 1990s when leptin was described (14). Later, it was shown that leptin, resistin, plasminogen activator inhibitor-1, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) had positive relationships to adiposity (15). Since they have some crucial roles in immune regulation, vascular function and adipocyte metabolism, adipocytokines are considered to be central players in the pathogenesis of metabolic syndrome, a cluster of clinical symptoms that include obesity and insulin resistance. Consequently, the regulation of body weight and obesity-related pathology is rapidly becoming a critical concern for public health experts and medical scientists worldwide (16). Most of the studies on the relationship of obesity and colorectal carcinogenesis are using obese animals (e.g. *db/db*, *ob/ob* or high-fat diet consumption mice). However, it is difficult to determine which adipocytokine is involved in colon carcinogenesis in obese animals because, in addition to several adipocytokines, there are a number of altered physiological factors in obese individuals.

Thus, in the present study, we decided to use chemically induced colon carcinogenesis in mice to quantify the serum levels of adipocytokines (17). In addition, the effects of dietary citrus nobiletin (NOB, Figure 1), a candidate chemopreventive agent against cancer in the colon (18,19), toward colon carcinogenesis and the serum level of adipocytokines in mice were investigated to elucidate its regulatory activities.

Materials and methods

Mice

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) were obtained at 5 weeks old and maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. On arrival, all mice were randomized and transferred to plastic cages (five mice per cage) and given free access to drinking water and a pelleted basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C). All mice were quarantined for 1 week before starting the experiments.

Chemicals

Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma Chemical Co. (St Louis, MO). Dextran sulfate sodium (DSS) with a molecular weight of 36 000–50 000 was purchased from ICN Biochemicals (Aurora, OH), dissolved in distilled water at a concentration of 1% (wt/vol) and then used to induce colitis. NOB (>98% purity) was obtained from Nard Chemicals (Hyogo, Japan). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and bovine serum were purchased from Gibco BRL (Grand Island, NY). Human recombinant leptin was obtained from R&D Systems (Minneapolis, MN). Antibodies directed against Pi-mitogen-activated protein kinase/extracellular signaling-regulated protein kinase kinase (MEK)1/2 (Ser217/221, #9121), Pi-extracellular signaling-regulated protein kinase (ERK)1/2 (Thr202/Tyr204), Pi-mammalian target of rapamycin (mTOR) (Ser2448, #2971), Pi-S6 (Ser240/244, #2215), Pi-eukaryotic initiation factor 4B (eIF4B) (Ser422, #3591), as well as horseradish peroxidase-conjugated anti-rabbit antibody (#7074), were obtained from Cell Signaling Technology (Beverly, MA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless specified otherwise.
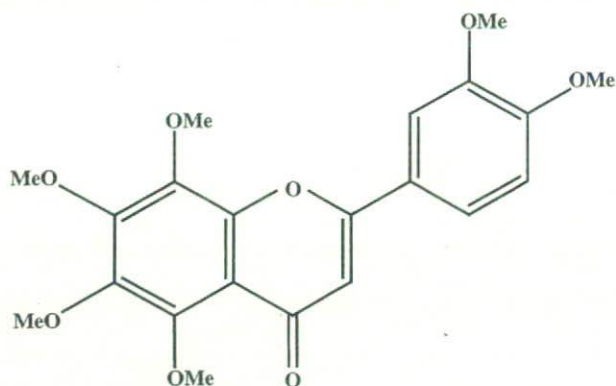
Abbreviations: AOM, azoxymethane; DMEM, Dulbecco's modified Eagle medium; DSS, dextran sulfate sodium; eIF4B, eukaryotic initiation factor 4B; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; IL-6, interleukin-6; MEK, mitogen-activated protein kinase/extracellular signaling-regulated protein kinase kinase; mTOR, mammalian target of rapamycin; NOB, nobiletin; Ob-R, leptin receptor; TNF-α, tumor necrosis factor-α.

Fig. 1. Chemical structure of NOB.

Fig. 2. Experimental protocol for AOM/DSS-induced carcinogenesis. Mice in groups 3 and 4 were given a single intraperitoneal injection of AOM (10 mg/kg body wt). Starting 1 week after the injection, they were administered 1% DSS in drinking water for 7 days (filled rectangle). Mice in groups 2 and 4 were given NOB (100 p.p.m.) in the diet from week 3.

Animal treatment

A total of 80 male ICR mice were divided into one control and three experimental groups (Figure 2). Group 1 served as an untreated control. Group 2 mice were given NOB (100 p.p.m.) in their diet starting from week 3 of the experiment. Mice in groups 3 and 4 were given a single intraperitoneal injection of AOM (10 mg/kg body wt) at the beginning of the experiment and then received 1% DSS in drinking water for 7 days starting 1 week after the injection. Further, group 3 mice were maintained on the basal diet throughout the study, whereas those in group 4 were given the same diet as group 2. The dose of NOB used was determined on the basis of our previous studies (18,19). The animals were sequentially euthanized on weeks 5, 10 and 20 as follows. Three mice each from groups 1 and 2 and five mice each from groups 3 and 4 were euthanized on weeks 5 and 10, whereas nine mice each from groups 1 and 2 and 15 mice each from groups 3 and 4 were euthanized on week 20. The mice were killed under ether anesthesia, and blood samples were immediately collected from the abdominal aorta, after which all organs were removed, with the colons flashed with phosphate-buffered saline, excised, measured in the length (from the ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed again with phosphate-buffered saline. The colons were macroscopically inspected, and whole colons were processed for paraffin embedding after being cut and fixed in 10% buffered formalin for at least 24 h. Histopathological examinations were then done on paraffin-embedded sections after hematoxylin and eosin staining. Colonic neoplasms were diagnosed according to the description by Ward (20). Tissues other than the colon were also evaluated histopathologically.

Clinical chemistry

The collected blood samples were used for clinical chemistry with measurements for triglycerides (Triglyceride E-test, Wako Pure Chemical Industries), adiponectin (Mouse/Rat Adiponectin ELISA Kit, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan), leptin (Quantikine Mouse leptin, ELISA/Assay Kit, R&D Systems), TNF-α (Quantikine Mouse leptin, TNF-α, ELISA/Assay Kit, R&D Systems) and IL-6 (Quantikine Mouse IL-6 ELISA Kit, R&D Systems, respectively) performed. Collected serum samples were examined without dilution to measure triglycerides, TNF-α and IL-6, whereas they were diluted 20- and 2000-fold for leptin and adiponectin measurements, respectively.

Cell culture

HT-29 human colon cancer cells and 3T3-L1 mouse pre-adipocytes were obtained from American Type Culture Collection (Manassas, VA). HT-29 and 3T3-L1 cells were maintained in DMEM supplemented with 10% FBS (HT-29) or 10% bovine serum (3T3-L1), as well as 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation

HT-29 cells (5×10^3/200 µl per well) were seeded into 96-well plates under the growth conditions described above. Twenty-four hours after seeding, the cells were serum starved for 24 h and then treated with leptin (0.01–10 nM) for various time periods (0–72 h), according to a method reported previously by Ogunwobi *et al.* (21), with some modifications. For suppressive experiments, cells were pretreated for 1 h with NOB (0, 10 and 100 µM) before leptin exposure. At various time points, cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.
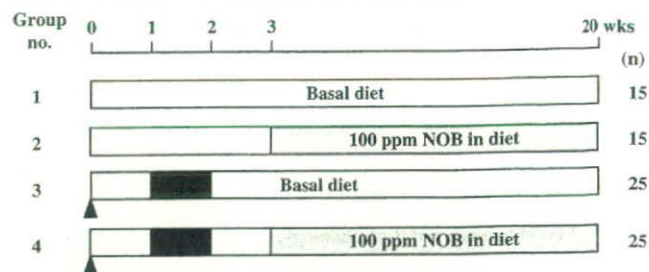
Intracellular lipid accumulation and adipocytokines secretion

The 3T3-L1 cells (1×10^4/200 µl per well) were seeded into 96-well plates under the growth conditions described above. After reaching confluence, they were incubated for an additional 24 h (designated as day 0). Then, adipocyte differentiation was induced by treatment with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 µM) and insulin (10 µg/ml), components of an Adipogenesis Assay Kit (Chemicon International, Temecula, CA), in DMEM containing 10% FBS for 48 h. The medium was then replaced by DMEM containing 10% FBS and insulin (5 µg/ml) and changed to fresh medium every 2 days, according to a method reported previously by Maeda *et al.* (22), with some modifications. On day 2, NOB (0, 10 and 100 µM) was dissolved in dimethyl sulfoxide and then added to DMEM containing FBS and insulin. The final concentration of dimethyl sulfoxide was 0.1%, which was found not to affect cell growth (data not shown). After 12 days, the medium was collected and subjected to enzyme-linked immunosorbent assay to determine the levels of leptin, adiponectin, IL-6 and TNF-α. The cells were stained with the Oil Red-O component of an Adipogenesis Assay Kit according to the manufacturer's instructions. Stained cells were viewed using an inverted microscope (Leica Microsystems, Tokyo, Japan) (original magnification 1:200) and images were captured with a digital camera system. Stained oil droplets in 3T3-L1 cells were extracted with dye extraction solution and then the absorbance of the extracts were measured at 490 nm.

Western blotting

Following treatment with NOB, 3T3-L1 cells were washed with phosphate-buffered saline twice and lysed in lysis buffer [10 nM Tris, pH 7.4, 1% sodium dodecyl sulfate, 1 mM sodium metavanadate (V)] and centrifuged at 3200g for 5 min. Denatured proteins (40 µg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h, the membranes were reacted with the appropriate specific primary antibody (1:1000) followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:1000). The blots were developed using ECL western blotting detection reagents.

Statistical analysis

Where applicable, data were analyzed using a Tukey–Kramer multiple comparison test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA), Fisher's exact probability test and Student's *t*-test (two sided), with $P < 0.05$ as the criterion of significance.

Results

General observations of mice

Throughout the study, dietary feeding with NOB did not cause clinically harmful symptoms including toxicity. The intake of water and food consumption (grams per day per mice) did not significantly differ among the four groups, and there were no marked changes in the mean relative liver weights and colon lengths (data not shown). In contrast, the mean body weight of group 3 was significantly higher ($P < 0.05$) than that of the groups 1 and 2, and NOB in the diet suppressed that increase by 56%, which was statistically significant (Table I). In parallel, the AOM/DSS treatment led to a notable increase in epididymal fat by 1.6-fold, whereas NOB tended to suppress that increase.

Table I. Body and epididymal fat weights and colon tumor formation in male ICR mice

Group no.	Treatment	Body weight (g)	Epididymal fat weight (g)	Incidence (no. of mice with neoplasms)			Multiplicity (no. of tumors/mouse)		
				Total (%)	Adenoma (%)	Adenocarcinoma (%)	Total	Adenoma	Adenocarcinoma
1	No treatment	41.8 ± 3.2^a	0.94 ± 0.42	0/6 (0)	0/6 (0)	0/6 (0)	0	0	0
2	100 p.p.m. NOB	43.0 ± 3.2^a	0.77 ± 0.65	0/6 (0)	0/6 (0)	0/6 (0)	0	0	0
3	AOM/DSS	49.3 ± 6.0^a	1.52 ± 0.79	5/10 (50)	3/10 (30)	$4/10 (40)^b$	2.1 ± 3.7	1.1 ± 2.0	1.0 ± 1.8
4	AOM/DSS + 100 p.p.m. NOB	45.1 ± 4.1	1.30 ± 0.61	1/10 (10)	1/10 (10)	$0/10 (0)^b$	0.1 ± 0.3	0.1 ± 0.3	0

Data are shown as the mean ± SD. Body and epididymal fat weights were measured using nine mice from groups 1 and 2 and 15 from groups 3 and 4. Colon tumor formation was analyzed using six mice from groups 1 and 2 and 10 from groups 3 and 4.
[a]Significantly different in Tukey–Kramer multiple comparison post test: $P < 0.05$.
[b]Significantly different in Fisher's exact probability test: $P < 0.05$.

Incidence and multiplicity of colonic neoplasms

We reported previously the significant effects of NOB toward AOM-induced aberrant crypt foci formation (18) and carcinogenesis (19) in rats. In the present study, we attempted to confirm its preventive ability in inflammation-associated colon carcinogenesis model mice and also examined its effects on the serum levels of adipocytokines. Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of mice in groups 3 and 4, which were shown to be tubular adenomas and adenocarcinomas in histopathological findings. As summarized in Table I, the mice in groups 1 and 2 did not develop neoplasms in any of the organs examined, including the colon. In contrast, group 3 had a 50% incidence of colonic tumors and 40% incidence of adenocarcinomas. In group 4, which received AOM/DSS and 100 p.p.m. of NOB in the diet, only a single colonic tumor developed in one mouse, which was shown in histopathological findings to be a tubular adenoma. Thus, the incidence of adenocarcinoma in group 4 (0%) was significantly lower than that in group 3 ($P < 0.05$). The multiplicity of colon adenomas in group 4 was also extremely lower than that in group 3.

Serum levels of leptin, adiponectin, IL-6, TNF-α and triglycerides

We assessed the serum levels of triglycerides and adipocytokines based on a previous report of a positive association of colon cancer with hypertriglycemia in *Apc* knockout mice (23). Interestingly, the serum concentrations of triglycerides, IL-6 and TNF-α in group 3 were elevated by 1.5–1.6-fold as compared with those in group 1, though the differences were not significant. Of note, the serum level of leptin in group 3 increased by 3.1–5.7-fold in a time-dependent manner (from 5 to 20 weeks) and was markedly suppressed by NOB (75–84%). When NOB was given by itself (group 2), it did not affect the level of leptin as compared with the control group.

Effects of leptin and NOB on cell proliferation

Leptin treatment (0.1–10 nM) for 24 h significantly increased HT-29 cell proliferation by 1.3–1.6-fold (Figure 4A) in a time-dependent manner (Figure 4B), which was consistent with previously reported findings (24,25). We also examined the effects of NOB on leptin-dependent and -independent cell growth. As shown in Figure 4C, NOB (10 or 100 μM) abolished leptin-enhanced cell growth, whereas it had no effects on cell proliferation when leptin was not added.

Effects of NOB on Oil Red-O staining and secretion of adipocytokines

We treated differentiated 3T3-L1 adipocytes with NOB (0.1, 1, 10 and 100 μM) to determine its effects on intracellular lipid accumulation and secretion of adipocytokines. Differentiated 3T3-L1 cells were notably loaded with lipid, as detected by Oil Red-O staining. NOB (100 μM) reduced the Oil Red-O staining level of 3T3-L1 cells to 40% (Figure 5A). Further, the flavonoid (1–100 μM) significantly reduced leptin secretion (61–100%, $P < 0.05$, Figure 5B) in a dose-dependent manner. However, it had no effect on the secretion of adiponectin, whereas IL-6 secretion was slightly increased by 10 μM of NOB. The

level of TNF-α in media was not detectable following any of the treatments (data not shown).

NOB inhibited leptin secretion partly through suppressed MEK1/2 phosphorylation

The mTOR, a Ser/Thr kinase, is considered to play a crucial role as the regulator of differentiation (26) and leptin secretion (27). We investigated the effects of NOB and rapamycin, an mTOR inhibitor, on the leptin secretion and phosphorylation status of molecules (Figure 6) involved in insulin-signaling pathway (mTOR, eIF4B, S6, Raf, MEK1/2 and ERK1/2). NOB (10 and 100 μM) significantly reduced leptin secretion as well as rapamycin (data not shown). The phosphorylation state of eIF4B and S6, both of which are substrates of mTOR, was abolished in rapamycin-treated cells, whereas NOB selectively decreased the phosphorylation state of only eIF4B. The differing results obtained with NOB and rapamycin led us to examine whether NOB affects the Raf/MEK/ERK pathway. Interestingly, the phosphorylation state of MEK1/2 and ERK1/2, but not Raf, was notably decreased in NOB-treated cells, whereas rapamycin did not affect those of both Raf and MEK1/2 but dramatically increased ERK1/2 phosphorylation. Proposed molecular mechanisms by which NOB suppresses leptin secretion are shown in Figure 7.

Discussion

In the present study, we demonstrated for the first time that serum leptin levels are profoundly increased in mice with chemically induced colon carcinogenesis. In addition, a citrus flavonoid, NOB, lowered not only those levels but also reduced colon tumor development, whereas other adipocytokines (TNF-α, IL-6 and adiponectin) and triglycerides did not significantly alter by the treatment. Further, NOB abolished leptin-stimulated human colon cancer cell proliferation and leptin secretion from insulin-treated adipocytes *in vitro* as well as the flavonoid rutin (28). Together, these results led us to hypothesize that an increased level of leptin promotes colon carcinogenesis in mice and that NOB is able to inhibit that, at least in part, through regulation of leptin levels.

Recently, obesity has become a point of focus in investigations conducted to identify dietary and lifestyle factors related to an increased risk of colorectal cancer (29). Metabolic stress resulting from obesity has been shown to be associated with increased levels of oxidative stress (30), inflammatory cytokines, insulin (31) and lipids (32,33). Of interest, Niho et al. (23,34) revealed a hyperlipidemic state in *Apc* gene-deficient mice, used as a model of human familial adenomatous polyposis, as compared with their wild-type counterparts. Our present findings regarding the serum level of triglycerides (Figure 3) are similar to those, though the difference did not reach statistical significance. This discrepancy may attribute to the differences in genetic backgrounds of the mice used and/or the experimental protocols. Obesity is driven by white adipose tissue, from which excess or reduced levels of adipocytokines are secreted (35). Adiponectin is the most abundant cytokine in adipocytes and has been reported to have antidiabetic and anti-inflammatory properties (36),
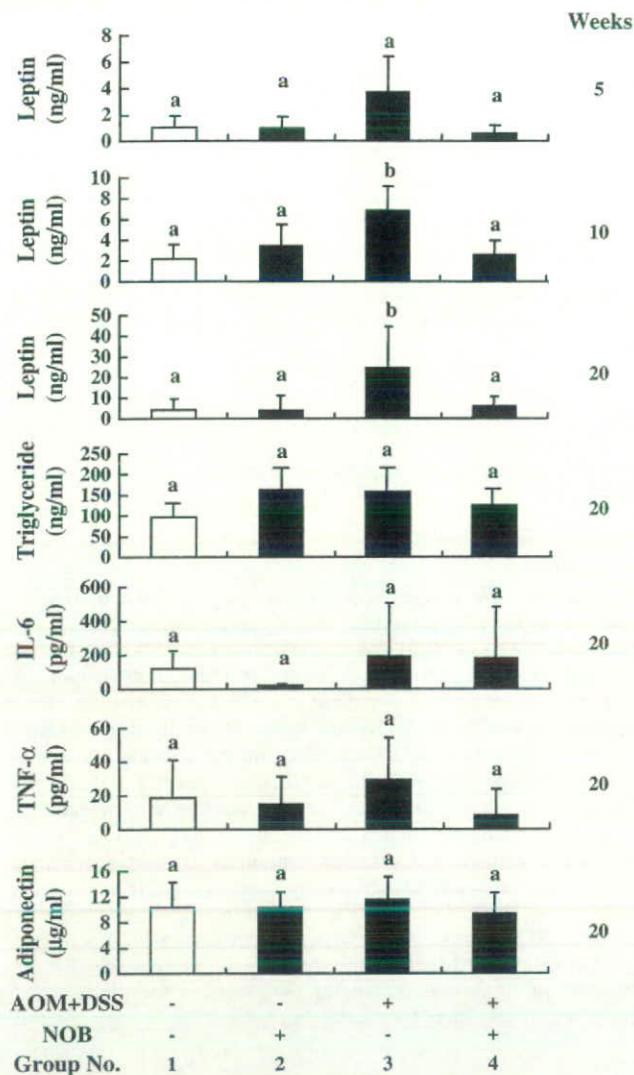
Fig. 3. Levels of serum leptin, triglycerides, IL-6, TNF-α and adiponectin in mice. Serum adipocytokine (leptin, IL-6, TNF-α and adiponectin) levels were quantified by enzyme-linked immunosorbent assay and triglycerides by a Triglyceride E-test. Values are shown as the mean ± SD (n = 3–15). Statistical analysis was performed using a Tukey–Kramer multiple comparison test and the data not sharing a letter, $P < 0.05$.

Fig. 4. Effects of leptin on proliferation of HT-29 human colon cancer cells and suppression by NOB. Twenty-four hours after seeding, cells were serum starved for 24 h and then treated with (**A**) leptin (0.01–10 nM) for 24 h (**B**) dimethyl sulfoxide (filled triangle) or 1 nM leptin (filled square) for different time periods (0–72 h) or (**C**) leptin in the presence of NOB (0, 10 and 100 μM) for 24 h. Cell numbers were determined using a Cell Counting Kit-8. Values are shown as the mean ± SD (n = 3). Statistical analysis was performed using a Student's t-test and the data not sharing a letter (a and b), $P < 0.05$ (at the same time point in panel B).

and low levels of adiponectin have been shown to be associated with an increased risk of colorectal cancer in humans (37). Several classical proinflammatory cytokines, e.g. TNF-α and to a large extent IL-6, are also secreted from adipocytes (38) and may participate in the regulation of obesity (39). In addition, epidemiological studies have revealed the roles of TNF-α and IL-6 in the onset of several types of cancer (40). However, there have been no results published regarding the hormonal role of leptin in chemically induced carcinogenesis in rodents.

Leptin is a 16 kDa protein encoded by the *ob* gene and was first revealed in 1994 as a regulator of body weight and energy balance, with its activities displayed in the hypothalamus (14). It is well known that serum leptin levels are highly elevated in obese individuals (41,42) and that leptin is secreted mainly by white adipocytes (43). C57BL/KsJ-*db/db* (*db/db*) mice have a defect in the leptin receptor (*Ob-R*) gene (44), which leads to leptin regulatory impairments of food intake thereby resulting in hyperinsulinemia, hyperglycemia and hyperleptinemia in subjects with extreme obesity (45). In the present study, the mean body and epididymal fat weights in AOM/DSS-treated mice were greater than those in control mice while NOB
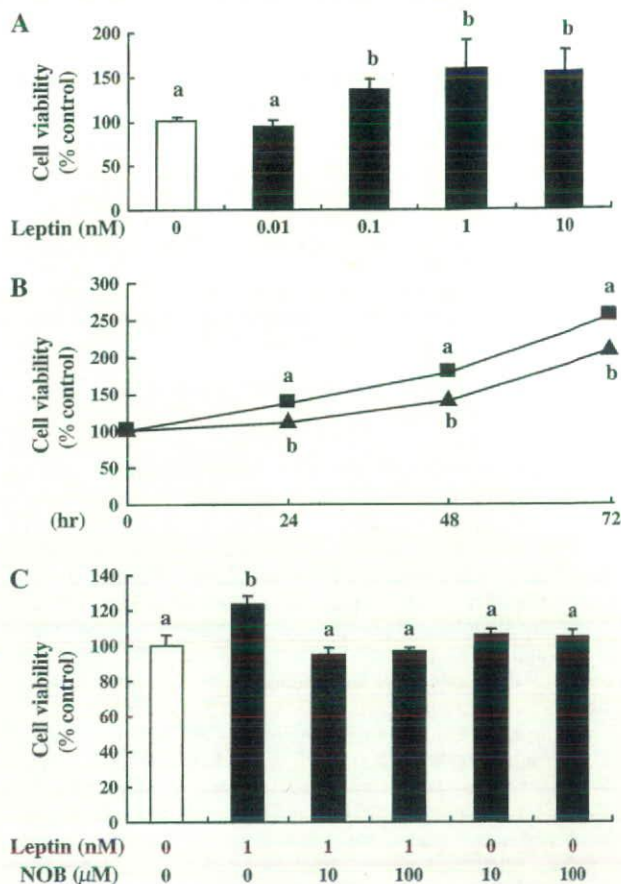
feeding decreased those (Table I). These results raise the possibility that the elevation of serum leptin levels seen in carcinogenesis model mice is in part due to increases in body and fat weights, though the underlying mechanism is unclear. On the other hand, several studies have reported that mesenteric adipose tissue in inflammatory bowel disease patients overexpress *leptin* mRNA (46,47). A DSS-induced colitis animal model is considered to be very reliable and useful for elucidating the mechanism underlying the onset of inflammatory bowel disease (48), thus DSS treatment may be associated with elevated serum leptin levels in AOM/DSS-treated mice. This issue is now being addressed in our laboratory. Our results (Figure 4) as well as those of several other studies (21,24,25) indicate that leptin acts as a mitogenic factor in cultured human colon cancer cells. However, it is well known that obese animals with elevated leptin levels, e.g. wild mice fed a high-fat diet and *db/db* mice, are highly susceptible to chemically induced carcinogenesis (49,50). Collectively, it is considered that obesity-associated colon carcinogenesis is partly mediated through a leptin-involved mechanism.

NOB (Figure 1), a polymethoxylated flavonoid predominant in citrus fruit peels (Figure 1) (51), has been reported to inhibit the proliferation of a variety of human cancer cell lines (52) and suppress colon carcinogenesis in rats (18,19). Although several reports have implied the preventive mechanism of NOB toward colon carcinogenesis, those results are not definitive. For example, NOB inhibited inducible nitric

oxide synthase and cyclooxygenase-2 expression in macrophages (53) and reduced prostaglandin E_2 levels in rat colonic mucosa treated with AOM (18,19). In the present study, we showed that dietary NOB decreased body and epididymal fat weights, which had been increased by treatment with AOM/DSS. These effects may contribute to its colon cancer preventive activities. In accordance with this notion, Saito *et al.* (54) recently reported that NOB enhanced both the differentiation and the lipolysis of adipocytes via activation of signaling cascades mediated by cyclic adenosine 3′,5′-monophosphate and cyclic adenosine 3′,5′-

monophosphate-responsive element-binding protein. Our findings showing that NOB reduced the accumulation of intracellular lipids (Figure 5A) were similar to those in that study.

In addition, it should be pointed out that NOB inhibited leptin-stimulated but not basal HT-29 colon cancer cell proliferation, though the mechanism is not fully understood. The biological activities of leptin are mediated through its receptor Ob-R, which consists of six splicing variants (Ob-Ra through Ob-Rf) (55). The long (Ob-Rb) and short (Ob-Ra) isoforms transduced leptin signals through ERK1/2- and c-Jun NH_2-terminal kinase 1/2-dependent pathways in human Kupffer and peripheral blood mononuclear cells (56). Also, leptin was reported to stimulate HT-29 cell proliferation through the activation of ERK1/2 and c-Jun NH_2-terminal kinase 1/2 (21,25). Importantly, we recently reported that NOB suppressed phorbol ester-induced activation of ERK1/2, c-Jun NH_2-terminal kinase 1/2 and c-jun in THP-1 human monocytic cells (57). Thus, this flavonoid may inhibit leptin-induced cell proliferation by disrupting mitogen-activated protein kinase pathways.

In adipocytes, mTOR is a master regulator of protein synthesis (58), adipose tissue morphogenesis (59) and leptin synthesis/secretion (60). Consistent with previous study, rapamycin significantly suppressed leptin secretion from 3T3-L1 cells (data not shown), and the suppressive effect may be due to the suppression of S6 and eIF4B, the substrates of mTOR (Figure 6). Meanwhile, in the present study, NOB suppressed the activation of eIF4B but not S6. Kawabata *et al.* (61) recently showed that eIF4B phosphorylation is dependent not only on mTOR but also on mitogen-activated protein kinase pathway. In this study, NOB notably suppressed the phosphorylation of MEK1/2 and ERK1/2, but not Raf (Figure 6). The MEK1/2 activation may be regulated not only by a conventional Ras/Raf signal pathway but also by their autophosphorylation (62). Furthermore, our findings are consistent with those by Miyata *et al.* (63) who reported that NOB inhibited the auto-phosphorylation of MEK1/2 without the affecting Ras and Raf activity in HT-1080 colon cancer cells. Together, it is likely that NOB inhibits phosphorylation of MEK1/2 and eIF4B for decreasing leptin release. Based on the different mode of actions between NOB and rapamycin, their combination may lead to additive or synergistic leptin suppression.
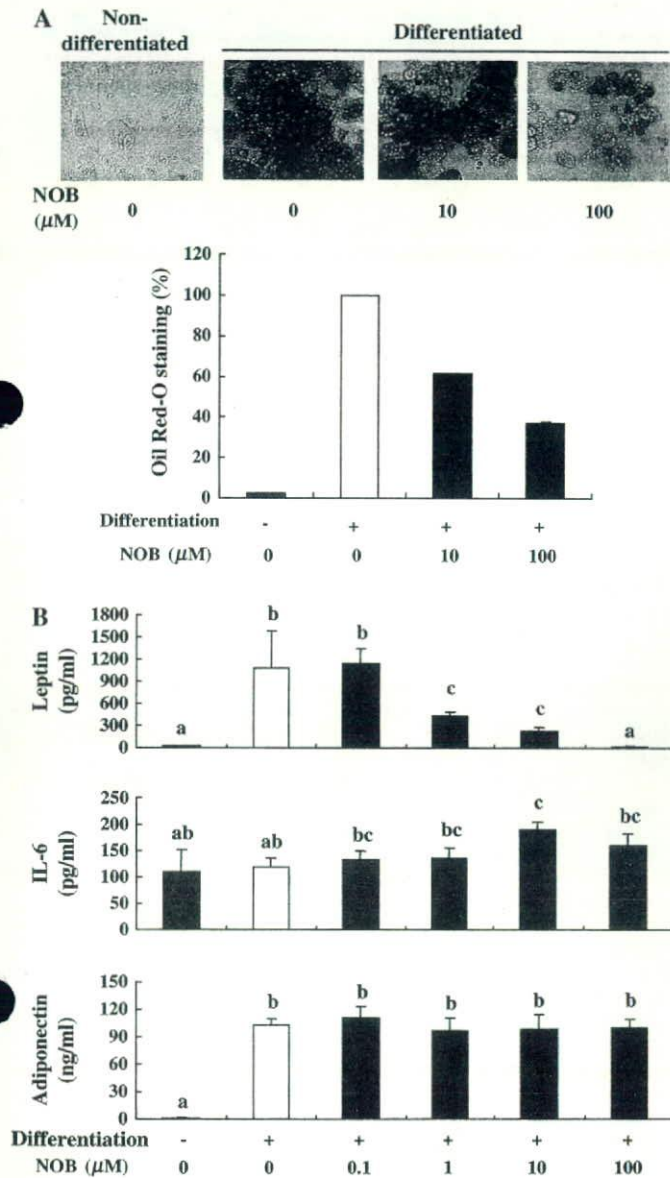

Fig. 5. Effects of NOB on Oil Red-O staining (A) and secretion of leptin, IL-6 and adiponectin from differentiated 3T3-L1 cells (**B**). 3T3-L1 mouse preadipocytes were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with dimethyl sulfoxide alone or various concentrations of NOB for 12 days and then the supernatants were removed for measurements of adipocytokines. The cells were washed twice with phosphate-buffered saline and stained with Oil Red-O. Stained cells were then viewed using an inverted microscope (Leica Microsystems) (original magnification 1:200). Leptin, IL-6 and adiponectin secretion were quantified by enzyme-linked immunosorbent assay. Values are shown as the mean ± SD ($n = 6$). Statistical analysis was performed using a Student's t-test and the data not sharing a letter, $P < 0.05$.
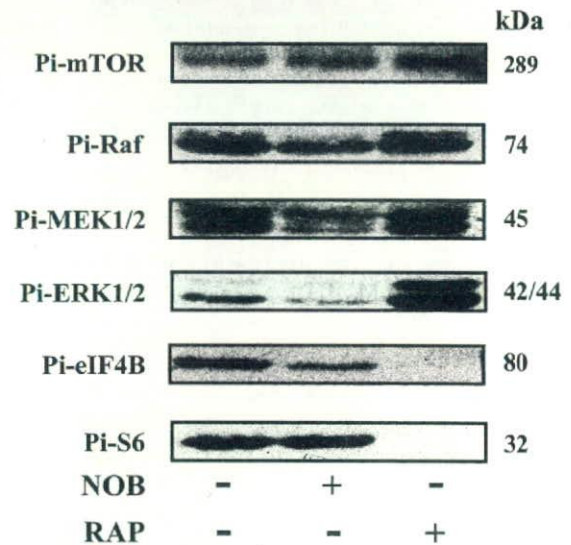

Fig. 6. Effects of NOB or rapamycin on mTOR-signaling pathway in differentiated 3T3-L1 cells. 3T3-L1 mouse pre-adipocytes (1×10^5 cells in 35 mm dish) were induced to adipocyte differentiation with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM) and insulin (10 μg/ml) in DMEM containing 10% FBS for 48 h. Differentiated 3T3-L1 cells were treated with dimethyl sulfoxide alone, various concentrations of NOB or 100 nM rapamycin for 12 days and then the supernatants were removed for measurements of adipocytokines. The cells were washed twice with phosphate-buffered saline and analyzed by western blotting using specific antibodies. RAP, rapamycin.
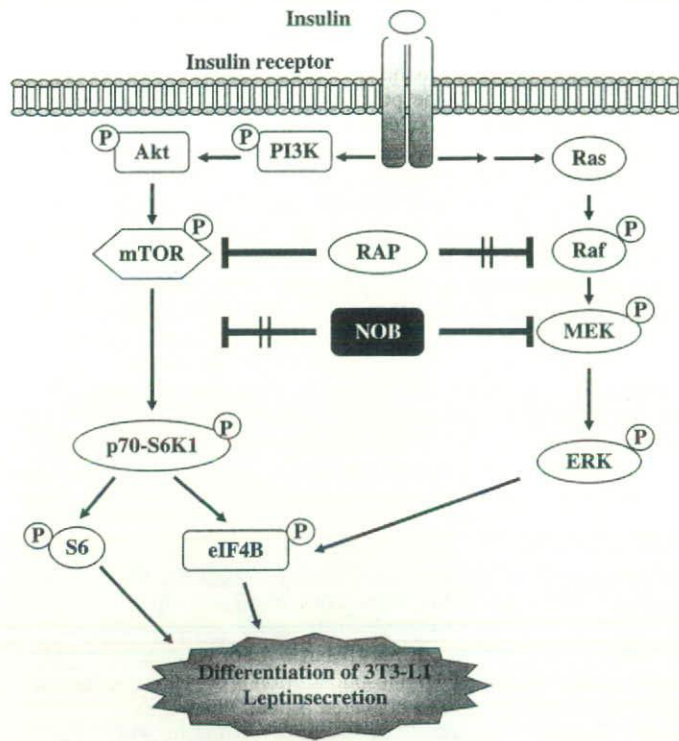
Fig. 7. Proposed schema of molecular mechanisms by which NOB suppresses leptin secretion from 3T3-L1 cells. Differentiation and protein translation are regulated by both Akt/mTOR and MEK/ERK-signaling pathway. mTOR and the downstream factors, such as S6 and eIF4B, are phosphorylated in a constitutive manner. Rapamycin inhibits mTOR and thereby suppressing the activity of downstream molecules for blocking leptin secretion. Meanwhile, NOB induces the dephosphorylation of MEK1/2 without affecting mTOR and Raf and reduces phosphorylation of eIF4B.

In conclusion, the present results suggest that the level of leptin in serum is related to colon carcinogenesis and dietary NOB suppresses carcinogenesis partly through regulation of this hormone. Although additional studies are necessary to confirm our speculation, synthetic drugs or food ingredients targeting leptin secretion and activities may be useful for regulating obesity-associated colorectal cancer development.

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A Tobacco-specific Carcinogen, NNK, Enhances AOM/DSS-induced Colon Carcinogenesis in Male A/J Mice

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Abstract. *To determine whether tobacco-derived carcinogens affect colon carcinogenesis, the effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on colon carcinogenesis were examined using an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. NNK (10 μmol) was administered to male A/J mice by a single intraperitoneal (i.p.) injection and then AOM (10 mg/kg body weight, i.p.) was given 1 week after NNK administration. One week later, the mice received 1.5% (w/v) DSS in their drinking water for 7 days. All animals were sacrificed at week 22 to examine the pathological lesions in the colon and lung. The incidence (80%, p<0.05) and multiplicity (4.0±3.6, p<0.05) of colonic tumors of the NNK+AOM+DSS group were significantly higher than that of the AOM+DSS group (incidence, 40%; and multiplicity, 1.2±1.7). The differences in incidence and multiplicity of lung tumors were insignificant between these two groups. Our findings may suggest that smoking increases the risk of inflammation-related colon cancer development.*

Many studies generally show a positive association between tobacco use and mortality from colorectal cancer (CRC) (1, 2), despite ambiguities in that some studies have not shown a relation between tobacco and CRC development (3). Incidence

Abbreviations: AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; CD, Crohn's disease; COX, cyclooxygenase; CRC, colon cancer; DSS, dextran sulfate sodium; HP, hyperplasia; LOX, lipoxygenase; nAChR, nicotinic acetylcholine receptor; NFκB, nuclear factor-κB; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; ROS, reactive oxygen species; UC, ulcerative colitis.

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Key Words: Cigarette smoking, NNK, colitis-associated colon cancer.

of malignant neoplasm was higher by 1.5- to 3-fold in smokers than in non-smokers (4, 5). Tobacco use is known to cause many types of cancer in different organs, including lung. Smoking is a risk factor of cancer development in organs not in direct contact with smoke. Approximately 20% of the large bowel cancers in men would be attributable to smoking (6). Compared with never smokers, current smokers showed a 4-fold increase in risk of hyperplastic and adenomatous polyps in the colon (7). Furthermore, the risk of hyperplastic polyps and adenomas remained for up to 10 years after they stopped smoking (7). In spite of such findings, however, experimental evidence that tobacco smoking is involved in the pathogenesis of CRC is insufficient. 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific nitrosamine derived from nicotine, a major alkaloid in tobacco smoke (8, 9) and has been implicated as a major cause of tobacco-associated lung cancer (9, 10). Ye *et al.* (11) reported that NNK stimulated cell proliferation *via* 5-lipoxygenase (LOX) and cyclooxygenase (COX)-2 expressions in SW116 human colon cancer cells. However, it has not been elucidated whether NNK affects colon carcinogenesis *in vivo*.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), frequently progress to CRC (12, 13). Smoking is an important environmental factor in the pathogenesis of IBD (14, 15) and differently affects CD and UC: smoking increases the risk for CD and decreases that of UC (14, 15). In an animal model of colitis, chronic treatment with nicotine exerted biphagic effects on trinitrobenzene sulfonic acid-induced colitis (16): lower doses of nicotine were protective, but higher doses were deleterious. These findings may suggest that smoking can influence inflammation in the large bowel and, therefore, possibly affect CRC development in the inflamed colon.

In the current study, we investigated whether NNK affects inflammation-related colon carcinogenesis using our mouse model with azoxymethane (AOM) and dextran sulfate sodium (DSS) (17, 18). This animal model for colitis-related CRC is useful to investigate the modifying effects of xenobiotics on colitis-related colon carcinogenesis, since large bowel
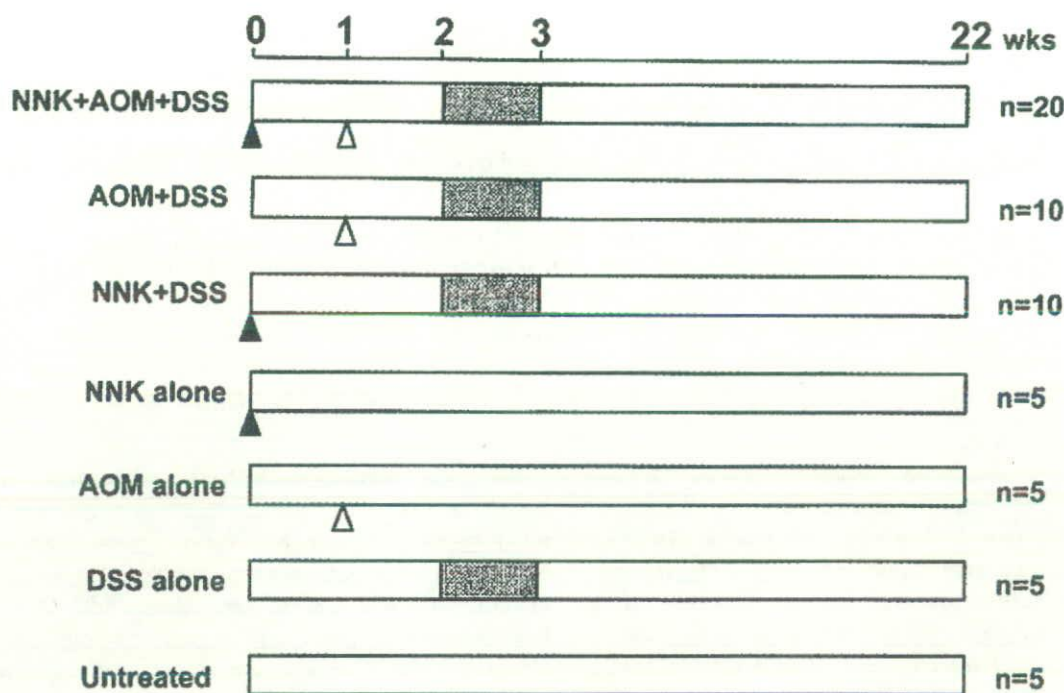
Figure 1. *Experimental protocol.* ▲ *NNK 10 μmol/mouse, i.p.;* △ *AOM 10 mg/kg bw, i.p.;* ▨ *1.5% DSS in drinking water.*

malignancies possessing biological alterations similar to those found in humans (18) develop within a short-term period.

Materials and Methods

Animals, chemicals and diets. Five-week-old male A/J mice were purchased from Charles River Japan (Tokyo, Japan). They were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guideline. All animals were housed in plastic cages (5 mice/cages) and given drinking water and a pelleted diet, CRF-1 (Oriental Yeast, Tokyo, Japan) *ad libitum*, under controlled conditions of humidity (50±10%), light (12/12 h light /dark cycle) and temperature (23±2°C). After arrival, they were quarantined for the first 7 days and then randomized by their body weights into experimental and control groups.

NNK was obtained from Toronto Research Chemical Inc. (Ontario, Canada). AOM was purchased from Sigma Chemical (St. Louis, MO, USA). DSS with a molecular weight of 36,000-50,000 (Cat. No. 160110) was purchased from MP Biomedicals, LLC (Aurora, OH, USA). DSS for induction of colitis was dissolved in water at a concentration of 1.5% (*w/v*).

Experimental procedures. A total of 60 male A/J mice were divided into 7 experimental and control groups (Figure 1). They were given a single intraperitoneal (*i.p.*) injection of NNK (10 μmol/mouse). After 7 days, animals were also treated with a single *i.p.* of AOM (10 mg/kg). Starting 7 days after AOM injection, 1.5% (*w/v*) DSS was given in the drinking water for 7 days. Experimental groups included: Group 1 (n=20) that received NNK, AOM and DSS; Group 2 (n=10) that were given AOM and DSS; Group 3 (n=10) was

treated with NNK and DSS; Groups 4, 5 and 6 (n=5, each group) were given AOM alone, NNK alone, and DSS alone, respectively; and Group 7 (n=5) was an untreated control group. At week 22, all mice were sacrificed to histopathologically investigate proliferative lesions in the lung and large bowel. At sacrifice, the lung and large bowel were removed and macroscopically inspected for the presence of tumors. The lung (all lobes) was measured and fixed in 10% buffered formalin for a least 24 h. After measuring the length of large bowels (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis and gently washed with saline. They were then cut along the vertical axis and fixed in 10% buffered formalin for a least 24 h. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections made from paraffin-embedded blocks. Colonic and lung tumors were diagnosed, according to Ward's (19) and Nikitin *et al's* descriptions (20), respectively.

Statistical analysis. The incidences among the groups were compared using chi-square test or Fisher's extract probability test with the GraphPad Instat Software (version 3.05; GraphPad software Inc., San Diego, CA, USA). Other measurements expressing mean±standard deviation (SD) were statistically analyzed using Tukey-Kramer multiple comparison post test (GraphPad Instat version 3.05). Differences were considered statistically significant at $p < 0.05$.

Results

General observation. Body weight gains during the study were comparable among the groups (data not shown). At sacrifice, the mean weights of body, liver, and lungs and

Table 1. *Mean body, liver, relative liver and lung weights, and mean colon length.*

Treatment	No. of mice	Body weight (g)	Liver weight (g)	% Liver weight (g)	Lung weight (g)	Colon length
NNK+AOM+DSS	20	27.5±3.6	1.46±0.20	5.31±0.32	0.43±0.07	13.7±1.0
AOM+DSS	10	28.1±1.5	1.59±0.13	5.66±0.35	0.52±0.08	15.1±0.6
NNK+DSS	10	27.7±1.2	1.58±0.17	5.70±0.49	0.53±0.07	15.2±1.0
NNK alone	5	30.9±1.8	1.72±0.18	5.56±0.39	0.56±0.07	16.0±0.6
AOM alone	5	28.4±1.8	1.47±0.15	5.19±0.54	0.50±0.06	16.1±0.6
DSS alone	5	28.1±1.0	1.53±0.09	5.44±0.31	0.45±0.04	15.8±0.3
Untreated	5	29.4±1.5	1.57±0.07	5.37±0.38	0.49±0.03	16.3±0.2

Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium.
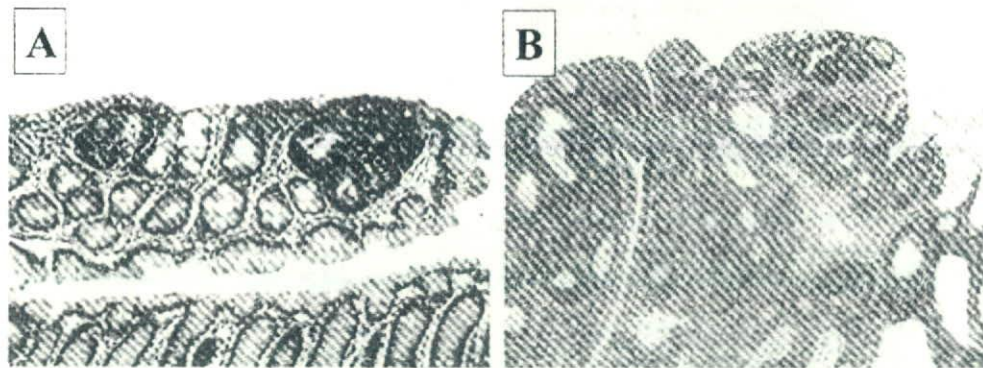

Figure 2. *Colonic neoplasms developed in mice from Group 1 that received NNK+AOM+DSS. A, tubular adenoma and B, tubular adenocarcinoma. H & E stain. Original magnification. (A) ×100 and (B) ×40.*
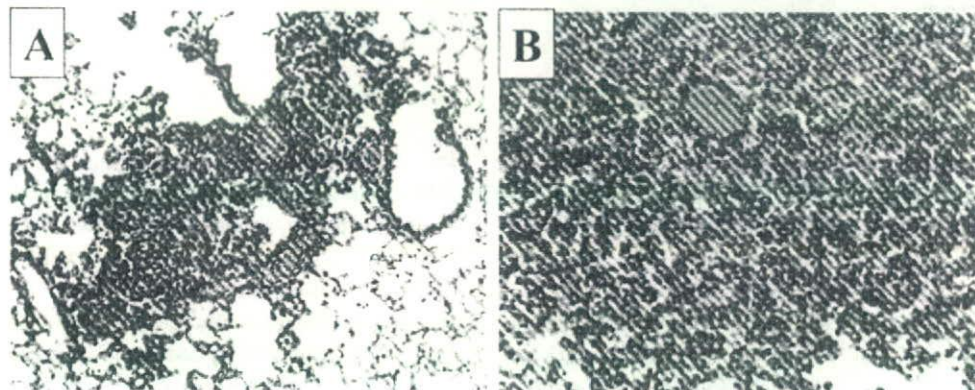

Figure 3. *Lung proliferative lesions developed in mice from Group 1 that received NNK+AOM+DSS. A, alveolar hyperplasia and B, adenoma. H & E stain. Original magnification. (A) ×40 and (B) ×100.*

colon lengths did not significantly differ among the groups, as shown in Table I.

Effects of NNK on colon carcinogenesis. Colonic tumors histologically diagnosed as adenoma (AD, Figure 2A) and/or adenocarcinoma (ADC, Figure 2B) developed in Groups 1 through 3, but not in Groups 4-7. Data on the incidence and multiplicity of colonic tumors are summarized in Table II. The incidence (80%) and multiplicity (4.00±3.58) of colonic tumors were the highest in Group 1, followed by

Table II. *Incidences and multiplicity of colonic tumors.*

Treatment	No. of mice	Incidence			Multiplicity (no. of tumors/colon)[a]		
		AD	ADC	Total	AD	ADC	Total
NNK+AOM+DSS	20	13[#]	16[*,#]	16[#]	1.80±1.88[##]	2.20±1.88[**,##]	4.00±3.58[**,##]
AOM+DSS	10	4	4	5	0.60±0.97	0.60±0.84	1.20±1.69
NNK+DSS	10	1	0	1	0.10±0.32	0	0.10±0.32
NNK alone	5	0	0	0	0	0	0
AOM alone	5	0	0	0	0	0	0
DSS alone	5	0	0	0	0	0	0
Untreated	5	0	0	0	0	0	0

AD, adenoma; ADC, adenocarcinoma. [a]Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium. [*]Significantly different from the AOM+DSS group by Fisher's exact probability test ($p<0.05$). [#]Significantly different from the NNK+DSS group by Fisher's exact probability test ($p<0.05$). [**]Significantly different from the AOM+DSS group by Tukey-Kramer multiple comparison post test ($p<0.05$). [##]Significantly different from the NNK+DSS group by Tukey-Kramer multiple comparison post test ($p<0.05$).

Table III. *Proliferative lesions of lung.*

Treatment	No. of mice	Incidence			Multiplicity (no. of proliferative lesions/lung)[a]		
		HP	AD	Total	HP	AD	Total
NNK+AOM+DSS	20	20	6	20	4.00±2.20	0.55±0.90	4.55±2.06
AOM+DSS	10	10	0	10	4.00±1.60	0	4.00±1.56
NNK+DSS	10	9	1	9	3.30±1.80	0.10±0.30	3.40±1.90
NNK alone	5	5	3	5	3.60±0.90	0.80±0.80	4.40±1.52
AOM alone	5	5	1	5	3.20±2.30	0.20±0.40	3.40±2.30
DSS alone	5	5	0	5	3.40±2.70	0	3.40±2.70
Untreated	5	5	0	5	4.00±1.00	0	4.00±1.00

HP, hyperplasia; AD, adenoma. [a]Data values are means±SD. NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; AOM, azoxymethane; DSS, dextran sulfate sodium.

Group 2 (50%, 1.20±1.69) and Group 3 (10%, 010±0.32). The incidence of colonic tumors of Group 1 was significantly greater than Group 3 ($p<0.05$), and the multiplicity of colonic neoplasms developed in Group 1 was significantly larger than Groups 2 ($p<0.05$) and 3 ($p<0.05$). Colonic ADC developed in Groups 1 (80% and 2.20±1.88) and 2 (40% and 0.60±0.84) with higher incidence and multiplicity in Group 1. The values were significantly greater than those of Groups 2 and 3 ($p<0.05$ for each comparison). Only one mouse of Group 3 developed a few colonic AD.

Effects of AOM/DSS on lung proliferative lesions. Lung proliferative lesions (hyperplasia and/or AD) developed in all mice in all groups. Data of the incidence and multiplicity of lung proliferative lesions are presented in Table III. Lung alveolar hyperplasia (Figure 3A) developed in all mice examined. The incidence and multiplicity of lung hyperplasia did not significantly differ among the groups. Lung AD (Figure 3B) developed in Groups 1, 3, 4, and 5, but not in mice of Groups 2, 6, and 7.

Discussion

In the current study, we investigated the effects of NNK on inflammation-associated colon cancer in male A/J mice. Our findings clearly showed that NNK enhances AOM/DSS-induced colon carcinogenesis, while treatment with AOM/DSS did not influence the occurrence of lung proliferative lesions induced by NNK. Our results were similar to epidemiological reports that current and ever smokers have increased odds of high-risk adenomas (21).

In the present study, we observed that colonic adenoma developed in a mouse of Group 3 that received NNK and DSS. Our series of investigation using an AOM/DSS mouse model used in this study demonstrated that the model can be used for detecting tumor initiation (22), tumor-promoting (23, 24) and chemopreventive (25) activities of xenobiotics in the colon. Our present findings suggest that NNK has initiation activity in the inflamed colonic mucosa, although the activity was weak. NNK is one of the nitrosamines metabolized from nicotine among the many components of

tobacco smoke (26). A/J mice are highly susceptible to chemically induced preneoplasms/neoplasms in the lung (27) and colon (28). Thus, this mouse strain seems suitable for use in a combined assay to evaluate potential modifying effects of xenobiotics on carcinogenesis in these tissues.

It is well known that cigarette smoking is a risk factor for carcinogenesis in a variety of tissues, such as those of the respiratory system (29) and digestive tract (30). In the colon, current and ever smokers have 2-fold increased odds of high-risk adenoma (21). Although it remains unclear whether smoking plays pivotal roles in colon carcinogenesis, it is likely that certain tobacco constituents, such as NNK, act as weak initiators in the carcinogenesis process. In this context, a recent report (31) identifying NNK as a high affinity ligand for neuronal nicotinic acetylcholine receptor (nAChR) comprised of α7-subunits, *i.e.* α7-nAChR, and expressed in human small cell lung carcinoma and endothelial cells is of interest. Furthermore, Ye *et al.* (11) reported that NNK enhanced α7-nAChR and its receptor mRNA expression in SW1116 human colon adenocarcinoma cells through increases in nuclear factor-κB (NFκB)-DNA binding activity and COX-2 and 5-LOX expression. Moreover, NNK treatment increased the level of intracellular reactive oxygen species (ROS) in SW1116 cells (11). NNK bioactivation leads to the production of ROS (32). ROS are known to activate NF-κB, which acts as a positive regulatory element of COX-2 expression (33). These results may indicate that α7-nAChR and ROS are involved in colonic tumorigenesis enhanced by NNK. Wong *et al.* (34) also showed that NNK stimulated HT-29 human colon cancer cell proliferation *via* activating mitogenic signal transduction pathway. This may be explained by the fact that NNK stimulated cell growth and affected the cell cycle. There are a few animal studies investigating the effects of tobacco-related carcinogens on colon tumorigenesis. Cigarette smoking enhanced colitis-related adenoma formation in mice (35) and COX-2 inhibitors reduced colitis-related colon tumorigenesis in mice (36, 37). Our results are in accordance with those reported by Liu *et al.* (35).

In the current study, AOM/DSS and DSS treatment (Groups 1 and 3 *vs.* Group 4) did not affect lung tumorigenesis induced by NNK. A tobacco-specific carcinogen, NNK can be stored in mammalian organisms. Importantly, the amount of NNK used in this study is high enough such that the total estimated doses to smokers and long-term snuff-dippers are similar in magnitude to the total doses required to produce cancer in laboratory animals (38, 39). This may be explained by the fact that after being given DSS in drinking water, DSS is mainly detected in Kupffer cells of the liver and macrophages of the mesenteric lymph nodes and large intestine, but not in the lung (40). Thus, DSS would not seem to be able to promote NNK-induced lung tumorigenesis. Another explanation for a lack of modifying effects of DSS on lung tumorigenesis is that DSS did not alter

CYP2A6 expression in the lung and liver, which is responsible for activation of NNK (41). AOM requires metabolic activation by CYP2E1 to exert its carcinogenic action (42). Both CYP2E1 and CYP2A6 are responsible for the metabolic activation of *N*-nitrosamines (43). Although we did not investigate the activity of CYP2A6 and CYP2E1 in the current study, it is important to investigate whether smoking and colitis affect expression of CYP2A6 and CYP2E1 in the colon and/or lung. In addition, it would be interesting to investigate the exact mechanisms, including immunomodulation (44) and cytokine expression (45), in order to determine how NNK contributes to colon tumorigenesis in the inflamed colon.

Although the effects of smoking on development of UC and CD are different (46), cigarette smoke exposure increases colitis-associated colonic adenoma formation in mice (35) and current and ever smokers have an increased risk of colonic adenoma (21). Our findings that a tobacco-specific carcinogen, NNK, influences inflammation-associated colon carcinogenesis in mice support these reports (21, 35).

Acknowledgements

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(−)-Epigallocatechin Gallate Suppresses Azoxymethane-Induced Colonic Premalignant Lesions in Male C57BL/KsJ-*db/db* Mice

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Abstract   Obesity and diabetes mellitus are risk factors for colon cancer. The activation of the insulin-like growth factor (IGF)/IGF-IR axis plays a critical role in this carcinogenesis. (−)-Epigallocatechin gallate (EGCG), the major constituent of green tea, seems to have both antiobesity and antidiabetic effects. This study examined the effects of EGCG on the development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) mice, which are obese and develop diabetes mellitus. Male *db/db* mice were given four weekly s.c. injections of azoxymethane (15 mg/kg body weight) and then they received drinking water containing 0.01% or 0.1% EGCG for 7 weeks. At sacrifice, drinking water with EGCG caused a significant decrease in the number of total aberrant crypt foci, large aberrant crypt foci, and β-catenin accumulated crypts in these mice, all of which are premalignant lesions of the colon. The colonic mucosa of *db/db* mice expressed high levels of the IGF-IR, phosphorylated form of IGF-IR (p-IGF-IR), p-GSK-3β, β-catenin, cyclooxygenase-2, and cyclin D1 proteins, and EGCG in drinking water caused a marked decrease in the expression of these proteins. Treating these mice with EGCG also caused an increase in the serum level of IGFBP-3 while conversely decreasing the serum levels of IGF-I, insulin, triglyceride, cholesterol, and leptin. EGCG overcomes the activation of the IGF/IGF-IR axis, thereby inhibiting the development of colonic premalignant lesions in an obesity-related colon cancer model, which was also associated with hyperlipidemia, hyperinsulinemia, and hyperleptinemia. EGCG may be, therefore, useful in the chemoprevention or treatment of obesity-related colorectal cancer.

Colorectal cancer is a serious health care problem worldwide. Recent evidence indicates that obesity and related metabolic abnormalities, including hyperglycemia, hyperlipidemia, and hyperleptinemia, are associated with an increased incidence of colorectal cancer (1–5). Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is also a key factor for the development of colorectal cancer (6). Insulin itself stimulates the growth of colon cancer cell lines while also promoting colorectal cancer tumor growth in animal model (7–10). Insulin resistance also causes alterations in the insulin-like growth factor (IGF)/IGF-IR axis, which is

involved in the development, progression, and metastatic potential of colorectal cancer (11–13). These reports suggest that hyperinsulinemia may be the essential consequence of obesity that increases the risk of colorectal cancer and, therefore, agents improving insulin resistance and targeting the IGF/IGF-IR axis might be able to inhibit the development of obesity-related colorectal cancer.

Numerous studies indicate that green tea catechins can exert anticancer and/or chemopreventive effects in various organ sites, including the colorectum (14, 15). Recent studies also show that green tea catechins possess antiobesity and antidiabetic properties (16). Experimental studies in rodents have shown that treatment with green tea or its constituents result in a significant reduction in body weight and, therefore, improve hyperlipidemia, hyperinsulinemia, and hyperleptinemia (17–19). These results suggest that long-term consumption of green tea is beneficial for the suppression of obesity and might reduce the risk of obesity-associated diseases, including the development of colorectal cancer. However, detailed studies whether green tea catechins can prevent the development of obesity-associated colorectal cancer have not yet been conducted.

Among the green tea catechins, (−)-epigallocatechin gallate (EGCG), the major biologically active component of green tea, is the most potent polyphenolic compound with respect