厚生労働科学研究費補助金（地球規模保健課題推進研究事業（国際医学協力研究事業））
分担研究報告書

アジア人のDNA付加体について

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研究要旨

地球規模での環境要因は、とくにnon-communicable disease代表的疾患であり、また近年、アジア地域などでも増加傾向のある悪性新生物の原因として極めて重要である。また、かなり明確な地域特性が環境中の化学物質とその地の高頻度のがんといった関係から認識される。本研究は、日中のともに胃がんの発生頻度の高い地域の胃粘膜から、網羅的付加体検索を行い、その修復機構、変異原性機構などを手がかりに本疾患と地域特性の関係を明らかに使用というものである。

A. 研究目的

本邦とアジアとくに中国の一地域における non-communicable disease の代表として 10 万人あたり 80 ほどの高い罹患率をもつ胃がんについて、その周辺粘膜の DNA 付加体の解析、同定によりアジアの環境による疾病の原因の一端を明らかにする。

B. 研究方法

非腫瘍部の胃粘膜から酸化防止剤存在下で DNA を抽出し、松田らの方法に従って adductome 解析（網羅的 DNA 付加体解析）を行った。その結果のなかで、7 種ほどの過酸化脂質由来の付加体について10の8乗塩基対あたりの数を算出し,両地域の症例について、クラスター解析、判別解析などを行う。また helicobacter については抵抗性遺伝子を PCR で同定した。

（倫理面への配慮）

介入をともなわない臨床研究に相当し、浜松医科大学ゲノム研究倫理審査委員会 91-23 による承認、また、南京大学倫理委員会および江蘇省からの Key laboratory の国際共同研究との承認を受けている。

C. 研究結果

対象とした付加体が内因性の炎症に関連することもあると思われるが、本邦の胃粘膜のほうがこれらの付加体の量はおおむね多かった。これらの付加体 profile によってどちらの地域からかが、判別できたので、がんの環境要因の決定の才に有力な手段であることがわかった。Helicobacter の頻度はやや本邦に多く、炎症由来の胃粘膜損傷が背景にあるということを裏付けるものであった。いっぽう、まだ同定されていない付加体が多数あり、そのなかには環境中の nitrosocompound など従来いわれているようなものあるいはそれに近いものがあるのではないかと研究を進める予定である。

DNA 損傷を変異原にもなる DNA 付加体として明確に同定することは、発がんにおいていわばもっともproximal な risk を体現すると考

えて良いと思う。これらの付加体を網羅的に認識したり、またそのゲノム上の位置を同定したりする問題が challenge として残っているが、今年度の結果は環境課題についてもこの adductome approach が有望であることを示した。

D. 結論

世界的にみて胃がんの頻度の高い本邦と、中国の一地域における胃粘膜に adductome 解析を行い、炎症性 adduct が本邦の胃粘膜に多いこと、さらに adductome profile で胃粘膜の origin(どちらの地域か)といったことが判別できることがわかり、環境発がんの暴露指標の評価法としてすぐれていると考えられた。

E. 健康危険情報　なし

F. 研究発表

G. 1. 論文発表

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A. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Hori M, Kitahashi T, Imai T, Ishigamori R, Takasu S, Mutoh M, Sugimura T, Wakabayashi K, Takahashi M	Enhancement of carcinogenesis and fatty infiltration in the pancreas in N-nitrosobis-(2-oxopropyl)amine-treated hamsters by high-fat diet.	Pancreas	40	1234-1240	2011
Matsubara S, Takasu S, Tsukamoto T, Mutoh M, Masuda S, Sugimura T, Wakabayashi K, Totsuka Y	Induction of glandular stomach cancers in Helicobacter pylori -infected Mongolian gerbils by 1-nitrosoindole-3-acetonitrile.	Int J Cancer	130	259-266	2011
Lim TH, Fujikane R, Sano S, Sakagami R, Nakatsu Y, Tsuzuki T, Sekiguchi M, Hidaka M	Activation of AMP -activated protein kinase by MAPO1 and FLCN induces apoptosis triggered by alkylated base mismatch in DNA.	DNA Repair	11	259-266	2012
Sugimura H, Tao H, Suzuki M, Mori H, Tsuboi M, Matsuura S, Goto M, Shinmura K, Ozawa T, Tanioka F, Sato N, Matsushima Y, Kageyama S, Funai K, Chou PH, Matsuda T	Genetic susceptibility to lung cancer.	Front Biosci (Schol Ed)	3	1463-1477	2011

Enhancement of Carcinogenesis and Fatty Infiltration in the Pancreas in *N*-Nitrosobis(2-Oxopropyl)Amine-Treated Hamsters by High-Fat Diet

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Objectives: Obesity is associated with increased pancreatic cancer risk, although the mechanisms have yet to be detailed. This study aimed to elucidate promotion of pancreatic cancer by obesity and hyperlipidemia.
Methods: Six-week-old female Syrian golden hamsters were treated with *N*-nitrosobis(2-oxopropyl)amine (BOP) and after 1 week were fed a high-fat diet (HFD) or standard diet (STD) for 6 or 17 weeks.
Results: Body weight and serum levels of lipids and leptin were significantly higher in the HFD than the STD group at 14 weeks of age. Pancreatic ductal adenocarcinomas developed only in the BOP + HFD group, with an incidence of 67% ($P < 0.01$) at 14 weeks of age. In addition, the multiplicity was 2-fold greater in the BOP + HFD group than in the BOP + STD group ($P < 0.05$) at 25 weeks of age. Pancreatic fatty infiltration was increased by BOP treatment and further enhanced by the HFD, correlating with progression of BOP-induced pancreatic ductal adenocarcinoma and up-regulated expression of adipocytokines and cell proliferation–related genes in the pancreas.
Conclusions: High-fat diet is shown to increase serum lipid levels and enhance fatty infiltration in the pancreas with abnormal adipocytokine production, which may accelerate and enhance pancreatic cancer.

Key Words: pancreatic cancer, hamster, fatty infiltration, hyperlipidemia, adipocytokine
(*Pancreas* 2011;40: 1234–1240)

Pancreatic cancer is one of the most lethal human cancers, with a 5-year survival rate generally less than 5%.[1] High incidences of pancreatic cancer are reported in developed countries, and the frequency increased from the 1950s to 1990s in Japan.[2] Epidemiological studies have shown that environmental factors such as cigarette smoking, dietary habits, and diseases such as chronic pancreatitis and diabetes are associated with pancreatic cancer risk.[2] Recent evidence suggests that obesity is also associated with an increased risk of pancreatic cancer.[3,4] Furthermore, elevated serum triglycerides (TGs) and a high intake of cholesterol may exert potential promotion on pancreatic carcinogenesis.[5,6] However, the promoting mechanisms of these factors on pancreatic carcinogenesis have yet to be completely elucidated.

As in the United States and in Europe, the prevalence of metabolic syndrome is growing rapidly in Japan and Southeast Asia. Because this may result in an increase in pancreatic cancer, it is important to elucidate the mechanisms for the purpose of cancer prevention. The Syrian golden hamster is in a hyperlipidemic state even under normal diet conditions because lipoprotein lipase activity in the liver is low compared with mice and rats.[7] The hamster is a unique model animal for the development of pancreatic ductal adenocarcinomas (PDACs) induced by the subcutaneous injections of *N*-nitrosobis(2-oxopropyl)amine (BOP).[8] Histopathologically, the induced lesions possess close similarities to pancreatic cancer in humans. Moreover, point mutations in codon 12 of the *K-ras* gene are frequently observed, and expression of the *fragile histidine triad gene* is aberrant in BOP-treated hamsters,[9,10] as is also observed in human PDACs.[11,12] The *p16* gene is one of the most frequently inactivated tumor suppressor genes in human PDACs,[13] and loss of *p16* expression has also been found in hamster PDAC lesions.[14]

We previously demonstrated that pioglitazone, a peroxisome proliferator–activated receptor γ ligand, improved hyperlipidemia and suppressed BOP-induced PDAC development.[7] In the present study, we examined whether aggravated hyperlipidemia with a high-fat diet (HFD) affects pancreatic carcinogenesis in BOP-treated hamsters and analyzed the possible involvement of serum lipids and adipocytokines.

MATERIALS AND METHODS

Animals and Chemicals

Five-week-old female Syrian golden hamsters were obtained from Japan SLC (Shizuoka, Japan) and acclimated to laboratory conditions for a week. They were housed 2 or 3 per plastic cage, with sterilized softwood chips as bedding, in an air-conditioned animal room, on a 12-hour light-dark cycle. As a standard diet (STD), CE-2 (crude fat, 4.8%; crude protein, 25.1%; total calories, 3.43 kcal/g [CLEA Japan, Tokyo, Japan]) was used, and 1 group of hamsters was fed Quick Fat diet (crude fat, 13.6%; crude protein, 24.2%; total calories, 4.06 kcal/g [CLEA Japan]) as the HFD. CE-2 contains soybean oils, and Quick Fat contains beef tallow as the main fats. Body weight and food consumption were measured weekly. Food and water

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This work was supported in part by Grants-in-Aid for Cancer Research (21-2-1); a grant of the Research Grant of the Princess Takamatsu Cancer Research Fund (08-24009); and a grant of the Third-Term Comprehensive 10-Year Strategy for Cancer Control, the US-Japan Cooperative Medical Science Program from the Ministry of Health, Labour and Welfare of Japan. M. Hori and S. Takasu are and T. Kitahashi was awardees of Research Resident Fellowships from the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive 10-Year Strategy for Cancer Control during the performance of the present research.
The authors declare no conflict of interest.

were available *ad libitum*. *N*-Nitrosobis(2-oxopropyl)amine was obtained from Nacalai Tesque (Kyoto, Japan).

Study of the Effects of an HFD on BOP-Induced Pancreatic Carcinogenesis in Hamsters

At 6 weeks of age, 87 of 117 hamsters were injected subcutaneously with BOP 4 times (on days 1, 3, 5, and 7) at a dose of 10 mg/kg body weight, whereas the remaining 30 received saline as vehicle controls. From 1 week after the last BOP treatment, 34 of 87 BOP-treated and 15 of 30 saline-treated hamsters were given the HFD. At 14 weeks of age, 11, 12, 9, and 9 hamsters in the BOP + STD, BOP + HFD, saline + STD, and saline + HFD groups, respectively, were killed under deep anesthesia. The splenic lobes of the pancreas of 3 hamsters in each group at 14 weeks of age were rapidly immersed in RNA Later (Ambion, Austin, Tex) for RNA protection and stored at −80°C until RNA extraction. At 25 weeks of age, the remaining animals were killed. Finally, 11, 12, 9, and 9 hamsters at 14 weeks of age and 42, 22, 6, and 6 hamsters at 25 weeks of age, respectively, were in BOP + STD, BOP + HFD, saline + STD, and saline + HFD groups, respectively. Blood samples were collected in all cases from the abdominal aorta. Visceral fat weights were assessed by weighing total adipose tissues surrounding uteri after dissection. At autopsy, the pancreas, heart, lungs, kidneys, liver, and bile duct were carefully examined macroscopically and then fixed in 10% phosphate-buffered formalin (pH 7.4). Each pancreas was carefully dissected from surrounding tissue and fixed after spreading on filter paper except for frozen pancreas for RNA extraction at 14 weeks of age. All paraffin-embedded organs were sectioned and stained with hematoxylin and eosin for assessment of histopathological features. Pancreatic lesions were histopathologically diagnosed as dysplasia and adenocarcinomas. Dysplasia corresponds to lesions in human that are called PanIN 3.The experimental protocol was in accordance with the guidelines for Animal Experiments in the National Cancer Center and was approved by the Institutional Ethics Review Committee for Animal Experimentation.

Levels of Blood Glucose, Serum Lipids, Adipocytokines, and Insulin

Blood glucose levels were measured using an automatic blood glucose meter (Medisafe-mini GR-102; Terumo, Tokyo, Japan). The levels of TGs and total cholesterol (TC) in the serum were analyzed using the FUJI Dri-Chem system (Fuji Film, Tokyo, Japan). Serum free fatty acid (FFA) levels were assayed by an enzymatic method (SRL, Tokyo, Japan). Serum leptin (B-Bridge International, Inc, Mountain View, Calif), adiponectin (R&D Systems, Inc, Minneapolis, Minn), and insulin (Millipore Corp, Billerica, Mass) were examined using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Quantification of Pancreatic Adipocytes

The percentage areas of adipocytes infiltrated in the splenic lobe relative to total areas of splenic lobe on each pancreatic section were calculated using Win ROOF image analysis software (Mitani Corp, Tokyo, Japan). Pancreatic splenic lobes without adenocarcinomas were chosen because pancreatitis associated with invasive growth of adenocarcinomas may damage parenchyma cells and cause further fatty infiltration.

Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from the pancreatic tissue using an RNeasy lipid tissue mini kit (Qiagen, GmbH, Hilden, Germany) combined with RNase-free DNase I (Invitrogen, Carlsbad, Calif), for degradation of genomic DNA. After RNA purification, aliquots of total RNA were subjected to reverse transcription (RT) reaction with oligo-dT and 9-mer random primers using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif). Each mRNA transcript was measured by a quantitative polymerase chain reaction (PCR) method with the DNA Engine Opticon 2 System (MJ Research, Waltham, Mass) and SYBR-Green chemistry (Bio-Rad Laboratories, Hercules, Calif) using the primer sets shown in Table 1. Data were calculated as ratios to 40S ribosomal protein S7 (RPS7) mRNA.

Statistical Analysis

The significance of differences in the incidences of dysplasia and PDAC was analyzed by the χ^2 test. Variation in other data was evaluated by the Student t test. $P < 0.05$ was regarded as significant.

RESULTS

Accelerated Body Weight Gain by the HFD

After the treatment of hamsters with a pancreatic carcinogen, BOP, at 6 weeks of age, the hamsters were fed an HFD or STD. The HFD group started to gain body weight at a greater rate than the STD group after 1-week feeding, and the difference

TABLE 1. The Primer Sequence Used for RT-PCR

Gene	Sequence
Leptin	
Forward primer (5′-3′)	CACCGGTTTGGACTTCATTC
Reverse primer (5′-3′)	CCACCACCTCTGTGGAGTAG
Plasminogen activator inhibitor 1	
Forward primer (5′-3′)	GTGCCCATGATGGCTCAGA
Reverse primer (5′-3′)	CGGGGCAGCCTGGTCATGTT
FASN	
Forward primer (5′-3′)	CTCAAGAAGGTGATCCGGGA
Reverse primer (5′-3′)	ACAGGGCTCACCAGGTTGTT
Monocyte chemoattractant protein 1	
Forward primer (5′-3′)	CTACAGCTTCTTTGGGACAC
Reverse primer (5′-3′)	AATGCCCCACTCACCTGCTG
IL-1β	
Forward primer (5′-3′)	CTTCATCTTTGAAGAAGAGC
Reverse primer (5′-3′)	TGTACAAAGCTCATGGAGAA
COX-2	
Forward primer (5′-3′)	AATGAGTACCGCAAACGCTT
Reverse primer (5′-3′)	GAGAGACTGAATTGAGGCAG
Insulin	
Forward primer (5′-3′)	GACCATCAGCAAGCAGGTCA
Reverse primer (5′-3′)	ACTGATCCACAATGCCACGC
IGF-1	
Forward primer (5′-3′)	GAGCTGGTGGACGCTCTTCA
Reverse primer (5′-3′)	TCAGATCACAGCTCCGGAAG
Cyclin D1	
Forward primer (5′-3′)	CCATGGAACACCAGCTCCTG
Reverse primer (5′-3′)	CGGTCCAGGTAGTTCATGGC
RPS7	
Forward primer (5′-3′)	CCAGAAAATCCAAGTCCGGC
Reverse primer (5′-3′)	AGTCCTCAAGGATGGCATCG

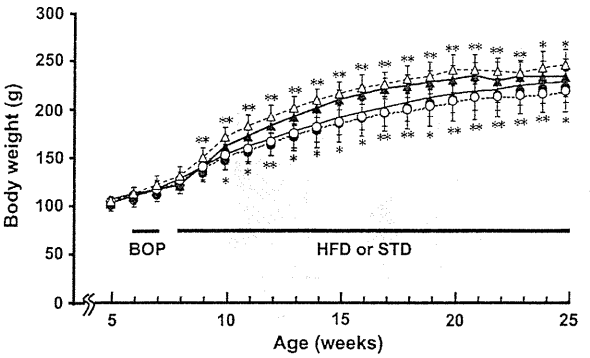

FIGURE 1. Body weight curves for the STD groups (circles) and the HFD groups (triangles) treated with BOP (closed symbols) or saline (open symbols). Data are means ± SD. Asterisks show the significance between the BOP + STD and BOP + HFD groups (upper) and between the saline + STD and saline + HFD groups (lower). *$P < 0.05$ and **$P < 0.01$ vs the respective STD group.

was obvious at 14 weeks of age (181 ± 9.6 vs 158 ± 13 g; $P < 0.01$; Fig. 1). Body weight curves reached plateaus at 25 weeks of age in the BOP + HFD and BOP + STD groups (214 ± 19 vs 203 ± 16 g; $P < 0.05$). Average values for food intake in the HFD and STD groups treated with BOP were almost the same, at 10.6 ± 0.3 g and 11.0 ± 0.8 g, respectively, whereas average calorie intake (kcal/hamster per day) was higher in the HFD group than in the STD group (43.1 ± 1.1 vs 37.9 ± 2.7; $P < 0.01$). Effects of BOP treatment on body weights and food intake were not observed.

Increases in the Levels of Serum Lipids With the HFD

At 14 weeks of age, the levels of serum lipids (TGs, TC, and FFAs) were significantly higher in the HFD than the STD groups, with and without BOP treatment (Table 2). Visceral fat weights in the HFD groups were significantly increased as compared with the STD groups. At 25 weeks of age, serum TG, TC, and FFA levels in the BOP + HFD group still remained higher than those in the BOP + STD group. However, increases of serum TC and FFA levels from 14 to 25 weeks of age were more apparent in the STD groups than in the HFD groups, resulting in smaller differences between the two. Interestingly, serum FFA levels were significantly higher in the BOP-treated than saline groups at 25 weeks of age.

Enhancement of Pancreatic Fatty Infiltration by the HFD

Fatty infiltration in hamster pancreatic tissues (Figs. 2A–D) was found to be characterized by infiltration of adipocytes into the intralobular spaces, without accumulation of fat within the parenchyma cells. Overall, adipocytes were diffusely present in the parenchyma tissues with particular accumulations around blood vessels. In the splenic lobes without PDACs, estimated percentage areas of adipocytes per area of pancreas were 4.4%, 7.4%, 7.9%, and 14.5% in the saline + STD, BOP + STD, saline + HFD, and BOP + HFD groups, respectively, at 14 weeks of age, with increase to 14.7%, 27.3%, 24.9%, and 41.9%, respectively, at 25 weeks of age (Fig. 2E). Fatty infiltration was increased by BOP treatment and also by the HFD feeding. In the BOP + HFD group, synergistic effects were observed.

Enhancement of PDAC Development and Increase of PDAC With Fatty Infiltration by the HFD

During the experiment, single animals in the BOP + STD and BOP + HFD groups died because of PDAC development at 19 and 21 weeks of age, respectively. The incidences and multiplicities of dysplasia as precancerous lesions and PDACs are summarized in Table 3. The incidence of dysplasia in the BOP + HFD group was higher than that in the BOP + STD group at 14 weeks of age and further increased at 25 weeks of age. Of note, PDACs developed only in the BOP + HFD group at 14 weeks of age at an incidence of 67%. The PDAC incidences at 25 weeks of age were similar at 80% and 86% in the BOP + STD and BOP + HFD groups, respectively, but the multiplicities of dysplasia and PDACs in the BOP + HFD group were 6- and 2-fold, respectively, of those in the

TABLE 2. Levels of Serum Lipids and Visceral Fat Weights in Hamsters

Group	Age, wks	No. Animals*	TGs, mg/dL	TC, mg/dL	FFAs, μEQ/L	Visceral Fat, g
BOP + STD	14	11	382 ± 87	188 ± 20	1058 ± 345	4.1 ± 2.2
BOP + HFD	14	12	510 ± 125†‡	269 ± 29†	1458 ± 320†	6.5 ± 1.8†
Saline + STD	14	9	402 ± 131	197 ± 38	1099 ± 319	3.3 ± 1.1
Saline + HFD	14	9	802 ± 196§	274 ± 90‖	1749 ± 338§	6.8 ± 2.3§
BOP + STD	25	10	344 ± 144	242 ± 23¶	1891 ± 306¶	10.1 ± 2.1¶
BOP + HFD	25	10	563 ± 126†	307 ± 64†	2214 ± 145†‡	11.3 ± 2.6¶
Saline + STD	25	4	284 ± 118	240 ± 30	1462 ± 292#	10.9 ± 2.2¶
Saline + HFD	25	3	546 ± 138§¶	317 ± 17§	1784 ± 234§	13.2 ± 2.0¶

Values are presented as mean ± SD.

*No. of animals assessed for visceral fat weight was 36 in the BOP + STD, 18 in the BOP + HFD, 6 in the saline + STD, and 6 in the saline + HFD groups at 25 weeks of age.

†$P < 0.01$ vs BOP + STD group.

‡$P < 0.01$ vs saline + HFD group.

§$P < 0.01$ vs saline + STD group.

‖$P < 0.05$ vs saline + STD group.

¶$P < 0.01$ vs the respective group at 14 weeks of age.

#$P < 0.05$ vs BOP + STD group.

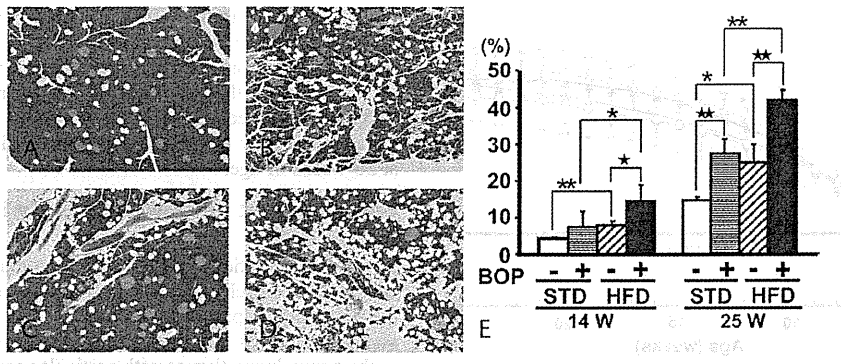

FIGURE 2. Effects of BOP treatment and the HFD feeding on fatty infiltration in the hamster pancreas. Representative data for hematoxylin-eosin–stained hamster pancreatic tissues in the saline + STD (A), BOP + STD (B), saline + HFD (C), and BOP + HFD (D) groups at 25 weeks of age are shown. Quantification of adipocyte areas per splenic lobe without PDAC was performed for each pancreas (n = 3–6, E). Open columns show the saline + STD group. Striped columns show the BOP + STD group. Hatched columns are for the saline + HFD group and closed columns for the BOP + HFD group. Original magnification ×40 for (A–D). *$P < 0.05$ and **$P < 0.01$ vs the respective value in the STD group. ★$P < 0.05$ and ★★$P < 0.01$ vs the respective value in the saline group.

TABLE 3. Incidences and Multiplicities of BOP-Induced Pancreatic Lesions

		No. Animals With Lesions, %		No. Lesions/Animal	
Group	**Age, wks**	**Dysplasia**	**Adenocarcinoma**	**Dysplasia**	**Adenocarcinoma**
BOP + STD	14	2/8 (25)	0/8 (0)	0.83 ± 0.98*	0
BOP + HFD	14	7/9 (78)†	6/9 (67)‡	1.00 ± 0.71	1.14 ± 0.69‡
BOP + STD	25	27/41 (66)	33/41 (80)	1.02 ± 0.91	1.66 ± 1.37
BOP + HFD	25	21/21 (100)‡	18/21 (86)	6.05 ± 2.84‡	3.19 ± 3.54†

*Mean ± SD.
†$P < 0.05$ vs BOP + STD group.
‡$P < 0.01$ vs BOP + STD group.

BOP + STD group. Figure 3 shows the sizes of PDACs at 25 weeks of age. In the BOP + HFD group, PDACs were rather larger than those in the BOP + STD group, especially the number of PDACs 2 to 3 mm in diameter being significantly higher.

Interestingly, adipocytes within PDACs were observed in large amounts in the BOP + HFD group at 25 weeks of age (Fig. 4A) but were a few in the BOP + STD group (Fig. 4B). Figure 4C shows the numbers of PDACs, classified according to the degree of fatty infiltration in BOP-treated hamsters. The HFD increased the number of PDAC with fatty infiltration within PDAC and its surrounding tissue. Differences between the BOP + HFD and BOP + STD groups were not observed in the numbers of PDAC without fatty infiltration.

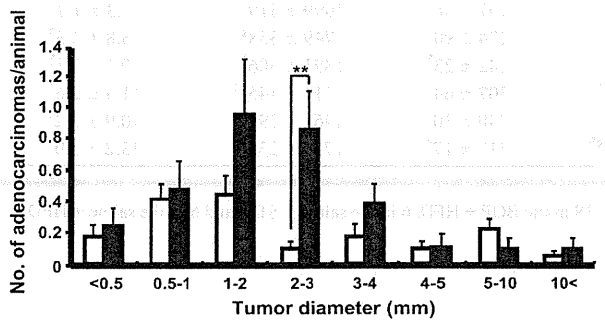

FIGURE 3. Size distributions of PDACs at 25 weeks of age. The numbers of each size of PDAC per hamster are given for the BOP + STD group (white bars) and BOP + HFD group (black bars). **$P < 0.01$ vs BOP + STD group.

In BOP-treated hamsters, it has been reported that carcinomas were induced in the bile duct, kidneys, liver, and lungs in addition to the pancreas.[8] At 14 weeks of age, no carcinomas were observed except for PDACs. At 25 weeks of age, cholangiocellular, hepatocellular, and lung carcinomas developed in the BOP + HFD group, and their incidences tended to be higher than in the BOP + STD group (cholangiocellular carcinomas: 71% vs 50%, hepatocellular carcinomas: 9.5% vs 7.1%, lung carcinomas: 33% vs 26%). No carcinomas were observed in the saline groups given either the HFD or the STD.

Increases in Serum Adipocytokine Levels and Pancreatic mRNA Expression Levels of Adipocytokine and Inflammatory- and Proliferation-Associated Genes by the HFD

As described above, differences of body weights and serum lipids between the BOP + HFD and the BOP + STD groups were obvious at 14 weeks of age, and PDACs were observed only in the BOP + HFD group at 14 weeks of age (Tables 2 and 3). Thus,

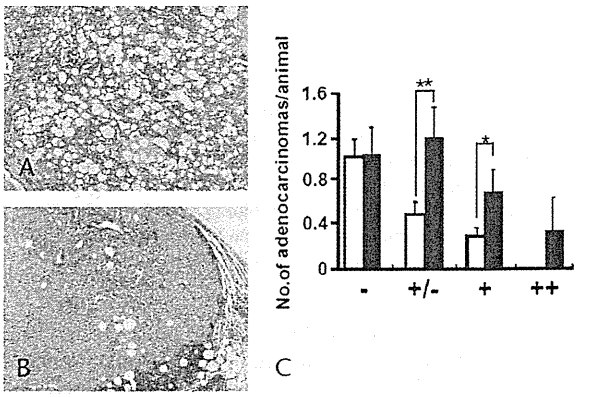

FIGURE 4. The levels of fatty infiltration within PDACs at 25 weeks of age. Representative data for fatty infiltration in PDACs in the BOP + HFD group (A) and BOP + STD group (B). The numbers of PDAC per hamster are given for the BOP + STD group (white bars) and BOP + HFD group (black bars) and are classified according to the degree of fatty infiltration per hamster (C). No fatty infiltration (−), fatty infiltration in surrounding tissue of PDAC (±), moderate fatty infiltration within PDAC (+; <10%), marked fatty infiltration within PDAC (++; ≥10%). Original magnification ×100 for (A and B). *$P < 0.05$ and **$P < 0.01$ vs BOP + STD group.

the serum levels of adipocytokines and insulin and mRNA expression levels of adipocytokines and inflammatory- and proliferation-associated genes in the pancreatic splenic lobes were compared between the BOP + STD and BOP + HFD groups at this time point. Serum leptin levels in the BOP + HFD group were 2 times higher than those in the BOP + STD group (21.7 ± 6.3 vs 10.4 ± 4.7 ng/mL; $P < 0.01$). In addition, serum adiponectin levels were higher in the BOP + HFD group than in the BOP + STD group (14.6 ± 2.0 vs 12.5 ± 1.5 ng/mL; $P < 0.05$). The levels of serum insulin (10.4 ± 3.1 vs 7.9 ± 3.0 ng/mL) and blood glucose (135 ± 25 vs 128 ± 31 mg/dL) did not significantly differ between the BOP + HFD and BOP + STD groups.

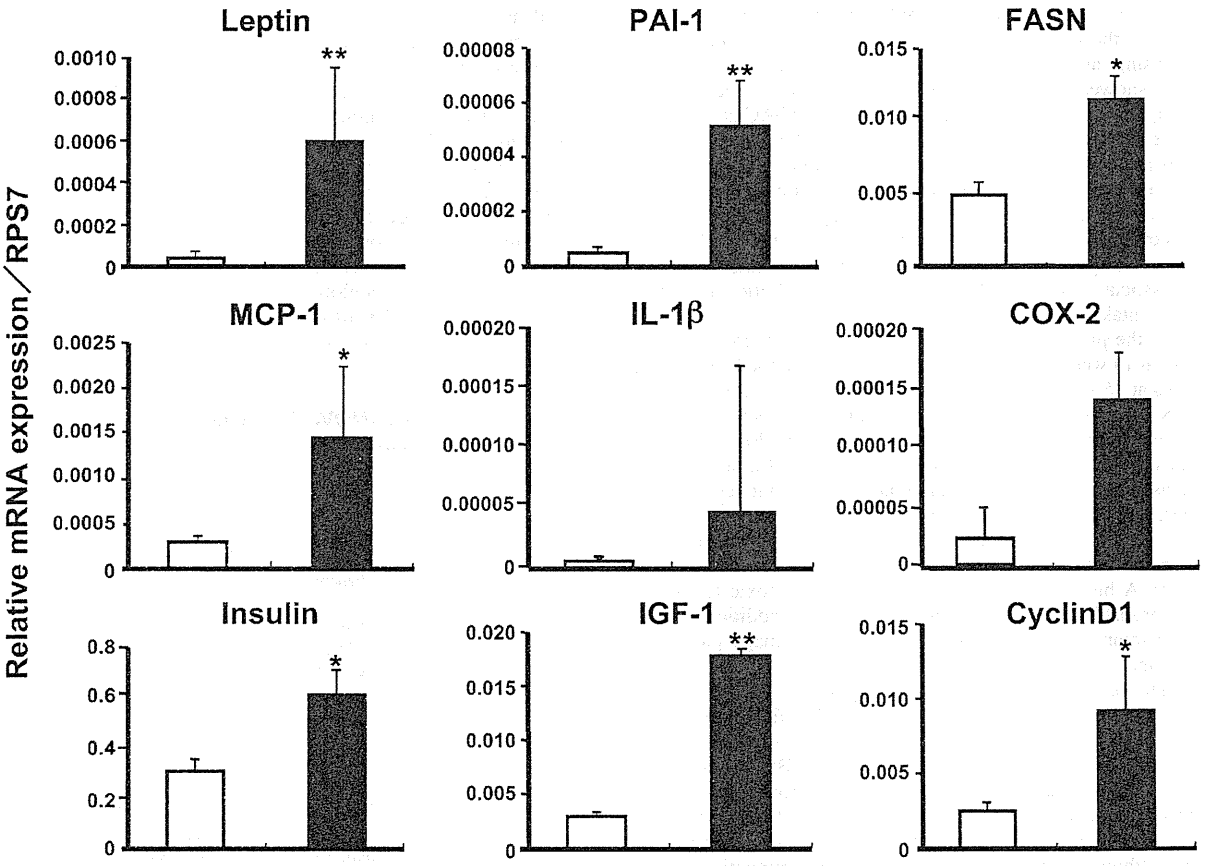

FIGURE 5. Expression levels of adipocytokines and inflammatory- and proliferation-associated genes in the hamster pancreas. Expression analysis of genes encoding adipocytokines and inflammatory- and proliferation-associated factors in the pancreatic tissue of BOP-treated hamsters fed either STD or HFD was conducted by real-time RT-PCR. The amounts of mRNA were normalized and shown relative to that of RPS7 mRNA in each sample with ranges determined by evaluating the expression: $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T$ + the SD of the $\Delta\Delta C_T$ value and $\Delta\Delta C_T$ − the SD of the $\Delta\Delta C_T$ value. Open columns, the BOP + STD group; closed columns, the BOP + HFD group. *$P < 0.05$ and **$P < 0.01$ vs the BOP + STD group.

mRNA levels in the pancreas for adipocytokines and lipid metabolism–related genes such as leptin, plasminogen activator inhibitor 1, and fatty acid synthase (FASN) were significantly higher in the BOP + HFD group than in the BOP + STD group (Fig. 5). Expression of inflammatory-related genes, including monocyte chemoattractant protein 1, IL-1β, and cyclooxygenase 2 (COX-2), also was increased or tended to be increased in the BOP + HFD group. The levels of mRNAs encoding growth-related genes such as insulin, insulin like growth factor I (IGF-I), and cyclin D1 were also elevated in the pancreas in the BOP + HFD group, indicating enhanced proliferation by the HFD.

DISCUSSION

In the present study, aggravated hyperlipidemia due to an HFD accelerated PDAC development with elevated serum levels of lipids and leptin. Moreover, pancreatic fatty infiltration and expression of adipocytokines and inflammation- and growth-associated genes in the pancreas were enhanced, indicating possible involvement in the mechanisms of promotion of PDAC development.

It has been reported that a high-corn-oil diet increased PDAC development in BOP-treated hamsters as compared with a low-corn-oil diet.[15] Furthermore, a diet containing beef tallow has been shown to increase PDAC development compared with a diet containing corn oil, although the amount of fat did not affect the yield of PDAC at 84 weeks of age.[16] Our present data using an HFD containing beef tallow as the main fat source showed early development of cancer at 14 weeks of age and higher yield of pancreatic dysplasia and PDAC at 25 weeks of age compared with the STD. Animal fats such as beef tallow are rich in cholesterol and saturated acids, and their intake has been shown to increase the levels of serum lipids.[17,18] Indeed, the HFD used in the present study made BOP-treated hamsters more hyperlipidemic compared with the STD. These findings suggest that the elevation of serum TG and TC levels was associated with PDAC development in addition to higher caloric intake by the HFD.

In the present study, serum FFAs was increased with HFD intake at 14 weeks of age and further enhanced in the BOP-treated groups at 25 weeks of age (Table 2). Moreover, the expression of FASN was observed to be enhanced in the pancreas tissues of the BOP + HFD-treated hamsters, indicating *de novo* FFA synthesis. In the case of Wistar rats, long-term intake of an HFD significantly increased body weight and serum lipids and then induced chronic pancreatic injury and microcirculatory disturbance.[19] Furthermore, in the present study, serum FFA levels correlated with pancreatic fatty infiltration in the BOP-treated groups (Table 2; Fig. 2). A high level of FFAs is reported to be toxic to various cells, probably because of peroxidation.[20,21] In addition to pancreatic injury induced by BOP, FFAs could damage pancreatic tissue and induce fatty infiltration.

The serum levels of leptin and adiponectin and visceral fat weights in the BOP + HFD group were higher than those in the BOP + STD group at 14 weeks of age (Table 2). Blood glucose levels did not significantly differ between the BOP + HFD and BOP + STD groups. Serum adiponectin is reported to correlate with insulin sensitivity.[22] Thus, it was indicated that insulin resistance was not caused in hamsters fed an HFD in the present study, although a hyperlipidemic state, hyperleptinemia, and accumulated visceral obesity were apparent. Leptin is reported to stimulate proliferation in colon,[23] breast[24] and prostate cancers,[25] and pancreatic β cells.[26] Furthermore, recent epidemiological studies have provided evidence that elevated circulating leptin levels are associated with obesity-related colon, breast, and prostate cancers.[27] Although the correlation between circulating leptin levels and cancer development remains inconclusive, in the present study, a correlation with hamster visceral fat and the incidence of PDAC was noted.

In addition, the expression levels of IL-1β and COX-2 also tended to increase in the BOP + HFD group (Fig. 5), and it has been shown that these are induced by leptin in rat brain and human endometrial cancer cells.[28,29] Overproduction of prostaglandin E_2 by COX-2 has been suggested to cause cell proliferation, angiogenesis, and antiapoptosis.[30,31] Other growth factors such as insulin and IGF-1 were also elevated in the pancreas of HFD-fed hamsters in the present study (Fig. 5). These growth factors have been reported to upregulate FASN.[32,33] Serum and tissue levels of IGF-1 may be increased in pancreatic cancer,[34] this factor being known to stimulate cell proliferation[35] with bidirectional crosstalk between leptin and IGF-1 signaling to promote cancer invasion.[36] Thus, the elevation of leptin, insulin, and IGF-1 in the hamster pancreas by the HFD in the present study could indicate promotion of cancer development through increases of inflammation- and growth-associated gene expression.

In humans, a high body mass index, elevated visceral fat weight and serum lipids, and diabetes mellitus have been shown to be the risk factors for pancreatic fatty infiltration.[37,38] Triglycerides and FFAs cause fatty infiltration in the pancreas in humans and animals, and obesity and diabetes mellitus are established risk factors for pancreatic cancer in humans.[3,4] Furthermore, pancreatic fatty infiltration has been clinically shown to promote dissemination of pancreatic cancer[39] and to increase the risk of pancreatic fistula after pancreaticoduodenectomy.[40] These available data suggest that it may alter the tumor microenvironment, enhance tumor spread, and be involved in progression of pancreatic cancer.

In conclusion, the present study demonstrated that an HFD accelerated PDAC development along with elevation of serum lipids and leptin. Moreover, enhanced pancreatic fatty infiltration and expression of adipocytokines in the pancreas were suggested to be involved in the promotion of PDAC development. Further clarification of mechanisms in detail and generation of evidence of involvement in humans are clearly warranted.

ACKNOWLEDGMENTS

The authors thank Ms Ruri Nakanishi and Mr Naoaki Uchiya for expert technical assistance.

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Induction of glandular stomach cancers in *Helicobacter pylori*-infected Mongolian Gerbils by 1-nitrosoindole-3-acetonitrile

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Helicobacter pylori (*H. pylori*) infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. We previously reported that Chinese cabbage contains indole compounds, such as indole-3-acetonitrile, a mutagen precursor. 1-Nitrosoindole-3-acetonitrile (NIAN), formed by the treatment of indole-3-acetonitrile with nitrite under acidic conditions, shows direct-acting mutagenicity. In the present study, NIAN administration by gavage to Mongolian gerbils (MGs) at the dose of 100 mg/kg two times a week resulted in three adduct spots (1.6 adducts/10^8 nucleotides in total), detected in DNA samples from the glandular stomach by ^{32}P-postlabeling methods. Treatment with six consecutive doses of 100 mg/kg of NIAN, two times a week for 3 weeks, induced well—and moderately—differentiated glandular stomach adenocarcinomas in the MGs at the incidence of 31% under *H. pylori* infection at 54–104 weeks. Such lesions were not induced in MGs given broth alone, broth + NIAN or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection.

Carcinogenesis

Gastric cancer is the second most frequent cause of cancer death worldwide.[1] Although gastric cancer has become a relatively rare cancer in North America and most Northern and Western European countries, it remains common in East Asia, Eastern Europe, Russia, and selected areas of Central and South America.[2] *Helicobacter pylori* (*H. pylori*) is a well-established major risk factor for gastric cancer,[3–5] and the prevalence of *H. pylori* infection in East Asia countries, including Japan and Korea is reported to be relatively high.[6,7] In addition, the risk of gastric cancer is increased with a high intake of various traditional salt-preserved foods.[3] In fact, pickled vegetable consumption is reported to increase gastric cancer risk in Japan and Korea.[8–10] In Korea, kimchi, commonly prepared with Chinese cabbage or radish, is a traditional and popular food, which contains high levels of nitrate (median 1550 mg/kg).[11] Furthermore, Chinese cabbage is well known as a pickled vegetable commonly consumed in Japan. Moreover, ingestion of nitrate, mainly from food, is suggested to correlate with mortality from gastric cancer.[12–14] Ingested nitrate is mainly converted to nitrite by bacteria in the oral cavity after secretion into saliva.[15] Carcinogenic *N*-nitroso compounds can be formed from nitrite and secondary amines under acidic conditions. Furthermore, direct-acting *N*-nitroso compounds, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)[16] and *N*-methyl-*N*-nitrosourea (MNU),[17] are known to induce cancer in the glandular stomach of experimental animals. Thus, it is suggested that *N*-nitroso compounds that are formed in the stomach under acidic conditions could be positively associated with the risk of gastric cancer. Nitric oxide, formed by nitric oxide synthase, is also reported to contribute to production of *N*-nitroso compounds.[18]

We have previously reported that treatments of various foodstuffs with nitrite under acidic conditions produce direct-acting mutagens towards *Salmonella* tester strains.[19,20] Among those foodstuffs, Chinese cabbage is shown to contain three indole compounds, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde as mutagen precursors. 1-Nitrosoindole-3-acetonitrile (NIAN), an *N*-nitroso-substituted compound formed by treatment of indole-3-

Key words: gastric cancer, *Helicobacter pylori*, Mongolian gerbil 1-nitrosoindole-3-acetonitrile, indole-3-acetonitrile

Abbreviations: DMSO: dimethyl sulfoxide; H&E: hematoxylin and eosin; *H. pylori*: *Helicobacter pylori*; MG: Mongolian gerbil; MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU: *N*-methyl-*N*-nitrosourea; NIAN: 1-nitrosoindole-3-acetonitrile.

Grant sponsor: Grants-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor, Welfare of Japan, and the U.S.-Japan Cooperative Medical Science Program

DOI: 10.1002/ijc.26020

History: Received 8 Oct 2010; Accepted 1 Feb 2011; Online 8 Mar 2011

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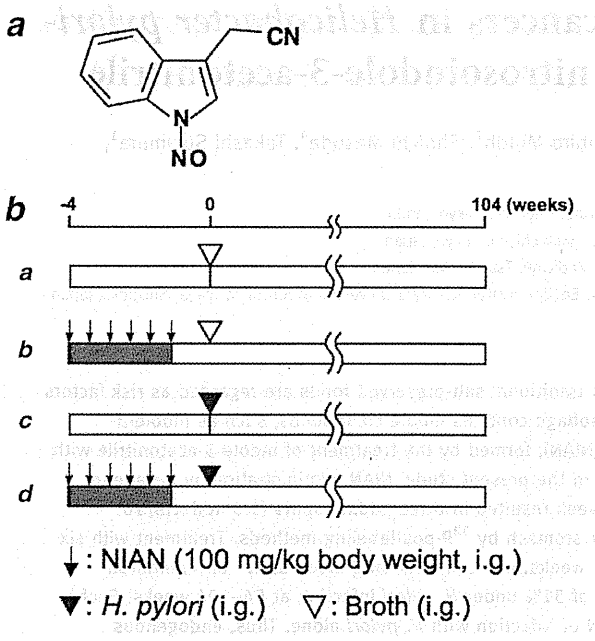

Figure 1. Chemical structure of NIAN and experimental protocol for the carcinogenicity study. (*a*) Chemical structure of NIAN. (*b*) Male 6-week-old MGs were orally administered NIAN (100 mg/kg) in 50% DMSO (groups B and D) or 50% DMSO alone (groups A and C) two times a week for 3 weeks. One week after the final administration, the animals were inoculated with *H. pylori* (ATCC 43504) (groups C and D) or sterilized broth (groups A and B).

acetonitrile with nitrite under acidic conditions, is a direct-acting mutagen in *S. typhimurium* and Chinese hamster lung cells,[20–22] and it is confirmed to form DNA adducts and to induce DNA single-strand scission in the rat glandular stomach.[23,24] Therefore, NIAN could play some role in gastric cancer development, as in the case of the well-known direct-acting mutagens, MNNG and MNU, in animal experiments.[16,17,25]

The Mongolian gerbil (MG) is reported to be susceptible to colonization by *H. pylori*, and *H. pylori* infection greatly enhances MNNG or MNU-induced gastric carcinogenesis in MGs.[26,27] Therefore, the MG is considered to be a useful animal model for evaluating the gastric cancer risk of direct-acting *N*-nitroso compounds, with or without *H. pylori* infection.

Chinese cabbage, containing nitrate and indole compounds, is commonly consumed in East Asian countries, including Japan, Korea and China, in which gastric cancer mortality is very high. In the present study, DNA adducts were detected with NIAN treatment in the glandular stomach of MGs, and the carcinogenicity of NIAN for gastric cancer *in vivo* was examined. The results clearly demonstrated that gastric cancer developed with a combination of NIAN administration and *H. pylori* infection in MGs. Possible involvement of indole compounds and nitrate derived from various foodstuffs, including Chinese cabbage, in gastric cancer development in humans is discussed.

Material and Methods

Materials

Indole-3-acetonitrile was purchased from Tokyo Food Techno (Tokyo, Japan), sodium nitrite from Wako Pure Chemical Industries (Osaka, Japan) and ammonium sulfamate from Kanto Chemical (Tokyo, Japan). Brucella broth was obtained from Becton Dickinson (Cockeysville, MD) and horse serum from Nippon Bio-Supply (Tokyo, Japan).

Preparation of NIAN

The chemical structure of NIAN is shown in Figure 1*a*. Indole-3-acetonitrile in 27 mM citrate-phosphate buffer (pH 3.0) was treated with 50 mM sodium nitrite for 1 hr at room temperature in the dark, as previously reported.[21] Nitrosation was stopped by addition of ammonium sulfamate at a final concentration of 50 mM. The reaction solution was filtered and the residue was washed with deionized water, then with *n*-hexane. The residual paste was dried and stored at −80°C until use. The preparation was >93% pure as judged by its UV absorbance on HPLC.

Bacterial culture

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was cultured in brucella broth supplemented with 10% heat-inactivated horse serum for 24 hr at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), as previously described.[28]

Animal treatment

Specific pathogen-free male, 6-week-old MGs (MGS/Sea, Kyudo, Fukuoka, Japan) were housed in a biohazard room, air-conditioned at 24°C ± 2°C and 55% humidity, on a 12 hr light–dark cycle and were allowed free access to commercial diet (CE-2; CLEA Japan, Tokyo, Japan) and water.

To analyze the formation of DNA adducts in the glandular stomach of MGs by NIAN treatment, NIAN was dissolved in 50% dimethyl sulfoxide (DMSO), and administered to three MGs by gavage of 0.5 ml solution, two times a week at a level of 100 mg/kg body weight. Two further MGs served as a control group receiving the solvent alone (0.5 ml). At 8 hr after administration of NIAN, both groups of animals were sacrificed under ether anesthesia, and their stomachs were resected and stored at −80°C until use. DNA was extracted by a standard procedure with enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v).

The protocol for long-term gastric carcinogenicity in MGs treated with NIAN + *H. pylori* infection is illustrated in Figure 1*b*. The animals were randomly divided into four groups (groups A–D). Groups A and C were given 50% DMSO without NIAN (0.5 ml) whereas groups B and D were orally administrated NIAN (0.5 ml, 100 mg/kg body weight) dissolved in 50% DMSO by gavage, two times a week for 3 weeks. At one week after the last administration, the

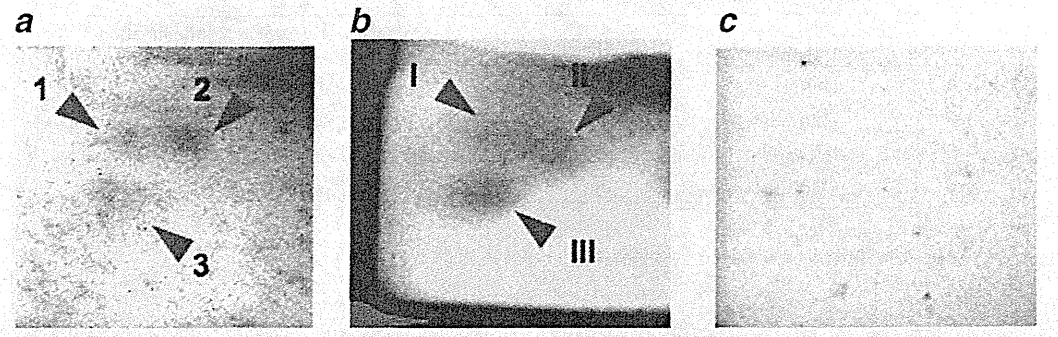

Figure 2. Autoradiograms of NIAN-DNA adducts in glandular stomach of MGs or calf thymus DNA treated with NIAN. Adducts were analyzed by ^{32}P-postlabeling method, as described in the Material and Methods. DNA samples were isolated from glandular stomach of MGs (*a*) or calf thymus DNA (*b*) after treatment with NIAN. DNA samples were also prepared from glandular stomach of MGs without NIAN treatment (*c*). Arrowheads indicate adducts. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

animals of groups C and D were given an intragastric inoculation of *H. pylori* broth culture (0.5 ml, 0.9 × 10^8 CFU/animal) whereas animals of groups A and B were given sterilized broth alone (0.5 ml).[28]

During the experiments, animals which became moribund or emaciated (<80 g body weight) were sacrificed. At 104 weeks after *H. pylori* infection, all surviving animals were sacrificed under ether anesthesia. At performance of necropsy, all tissues were carefully checked macroscopically and the stomachs and major organs were removed and assessed for macroscopic lesion development. Effective numbers of animals were defined as those surviving until week 54 of the study, when gastric tumors were observed for the first time. In addition, in the *H. pylori*-infected groups, the animals developing gastritis observed on histological examination were regarded as effective. The percentages of gastritis-bearing animals by the single inoculation of *H. pylori* were 62% for group C and 76% for group D, being similar to those previously reported.[27] All animal experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

Detection of DNA adducts by ^{32}P-postlabeling method

Calf thymus DNA (0.5 mg, Sigma, St. Louis, MO) treated with NIAN (3 mg) for 12 hr under neutral conditions was used for authentic NIAN-DNA adducts.[23] DNA samples from the glandular stomach of MGs and calf thymus DNA samples were digested with micrococcal nuclease and phosphodiesterase II, and subjected to ^{32}P-postlabeling analysis using the same procedure as described previously[23] except with solvent systems for two-dimensional development. The solvent system consisted of buffer A (4.0 M lithium formate, 7.7 M urea, pH 3.5) from bottom to top, and buffer B (0.90 M lithium chloride, 0.45 M Tris-HCl, 7.7 M urea, pH 8.0) from left to right, followed by 1.7 M sodium phosphate buffer, pH 6.0, from left to right, with 3.5 cm filter paper. Adducts were detected with a Bio-Image Analyzer (BAS 3000; Fuji Photo Film, Tokyo, Japan) after exposing the TLC sheets to Fuji imaging plates. Relative adduct labeling was determined by the methods of Reddy *et al.*,[29] and values were calculated as averages using data from three assays.

Histological examination

All excised stomachs were opened along the greater curvature and washed twice with saline, then fixed in 10% neutral-buffered formalin. The fixed stomachs were sliced along the longitudinal axis into 9–12 strips of equal width, and routinely processed to sections stained with hematoxylin and eosin (H&E). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System,[30] by scoring the infiltration of neutrophils and mononuclear cells. Other organs, in which macroscopic lesions were observed, were also fixed in 10% neutral-buffered formalin and routinely processed to sections stained with H&E for histological examination.

Statistical analysis

The significance of differences in quantitative data for gastric inflammation, gastric adenocarcinoma and tumors of other organs was analyzed by Fisher's exact test. Data for stomach wet weight and inflammation score were examined using Tukey's multiple comparison test. Significance was concluded at $p < 0.05$.

Results

DNA adduct formation by NIAN administration in the glandular stomach of MGs

To confirm the formation of NIAN-DNA adducts in the glandular stomach of MGs, NIAN was injected two times a week at a dose of 100 mg/kg by gavage, and then analyzed by ^{32}P-postlabeling method. Three adduct spots were observed in DNA samples derived from NIAN-treated animals (Fig. 2*a*). The adduct levels were 0.3 for adduct 1, 1.1 for adduct 2, 0.2 for adduct 3 and 1.6 adducts/10^8 nucleotides

Table 1. *H. pylori* infection induced-gastritis in MGs

Group	Treatment	Effective No.	Stomach wet weight (g)	Inflammation score
A	Broth	15	0.647 ± 0.097	0
B	NIAN + Broth	22	0.631 ± 0.094	0
C	*H. pylori*	18	1.432 ± 0.445*	2.22 ± 0.43*
D	NIAN + *H. pylori*	26	1.483 ± 0.445*	2.38 ± 0.64*

*$p < 0.01$ versus group A and B.
Values for results are expressed as averages ± SD.

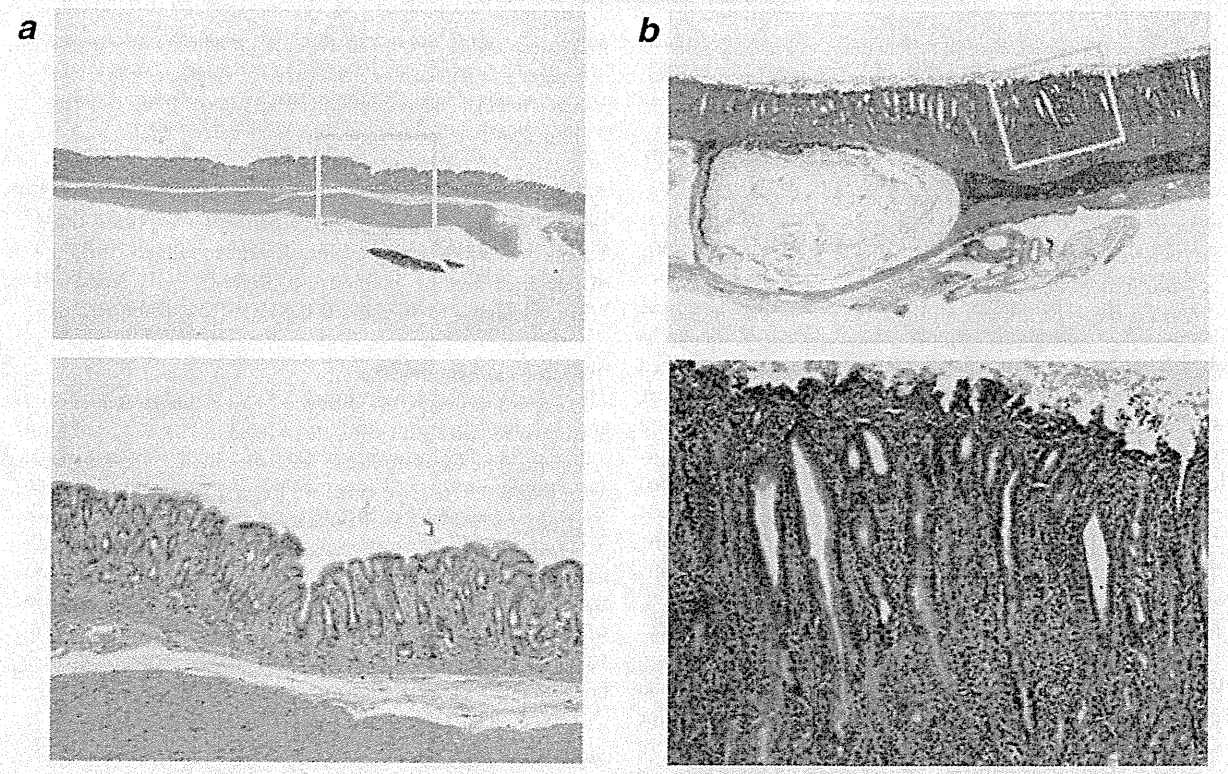

Figure 3. Macroscopic and microscopic views of gastritis in MGs infected or uninfected with *H. pylori*. (*a*) Normal gastric mucosa in group A. (*b*) Severe infiltration of many inflammatory cells with development of heterophilic proliferative glands in group C; H&E staining, ×40. Yellow boxes are shown at greater magnification below, ×200.

in total. This TLC pattern was similar to that in the *in vitro* reaction of calf thymus DNA with NIAN (total adduct level of 4.8 adducts/10^7 nucleotides, Fig. 2*b*). In the case of DNA samples derived from control animals, no adduct spots were seen on the TLC sheets (Fig. 2*c*).

Macroscopical and microscopical observation of *H. pylori*-induced gastritis in MGs

MGs were sacrificed until 104 weeks after *H. pylori* infection, and gastric disorders were analyzed. Stomach wet weights and gastric inflammation scores are shown in Table 1. Macroscopically, edematous thickening with hemorrhagic spots was apparent in the gastric mucosa in *H. pylori*-infected MGs (groups C and D), but not in animals uninfected with *H. pylori* (groups A and B). The stomach wet weight, reflecting edematous thickening, in animals infected with *H. pylori* (groups C and D) was significantly increased compared with that of animals not infected with *H. pylori* (groups A and B) ($p < 0.01$). No significant differences of stomach wet weight were detected between groups A and B and also between groups C and D.

Microscopically, gastritis, featuring infiltration of many inflammatory cells, and hyperplastic change of glandular epithelium, and erosion were observed in the pyloric regions of

Table 2. Incidence of glandular stomach adenocarcinoma in MGs

Group	Treatment	Effective No.	No. of animals with glandular stomach adenocarcinoma (%)		
			Total	Well dif.	Moderately dif.
A	Broth	15	0 (0)	0 (0)	0 (0)
B	NIAN + Broth	22	0 (0)	0 (0)	0 (0)
C	*H. pylori*	18	0 (0)	0 (0)	0 (0)
D	NIAN + *H. pylori*	26	8 (31)*	7 (27)	1 (4)

Well dif., well differentiated adenocarcinoma; Moderately dif., moderately differentiated adenocarcinoma.
*$p < 0.05$ versus group A and C and $p < 0.01$ versus group B.

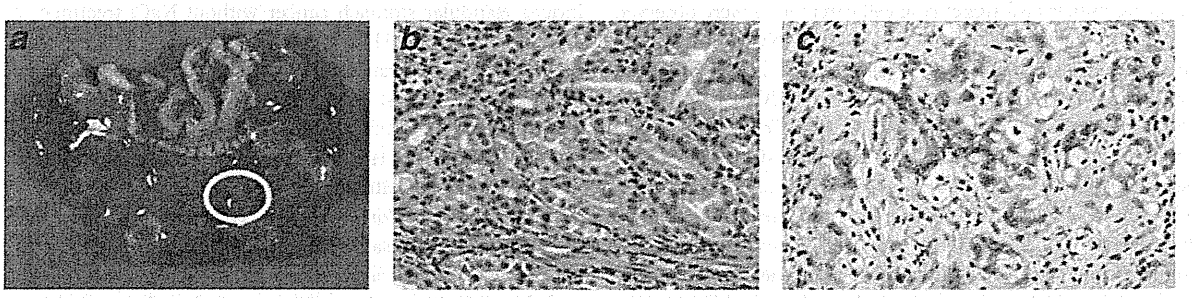

Figure 4. Histological findings of gastric adenocarcinoma in the animals treated with both NIAN and *H. pylori*. (*a*) Typical macrograph of a stomach. The yellow circle shows the suspected lesion of gastric cancer. (*b*) Well differentiated adenocarcinoma. (*c*) Moderately differentiated adenocarcinoma. (*b* and *c*) H&E staining, ×400.

the animals infected with *H. pylori* (groups C and D) (Fig. 3). Heterotopic proliferative glands, whose development is related to severe gastritis in *H. pylori*-infected MGs, were sometimes observed in *H. pylori*-infected groups (groups C and D). No gastritis was found in animals not infected with *H. pylori* (groups A and B). The gastric inflammation score in *H. pylori*-infected animals was significantly increased compared with that of animals uninfected with *H. pylori* ($p < 0.01$). There were no significant differences of gastric inflammation score between groups C and D.

Development of glandular stomach adenocarcinomas in MGs treated with both NIAN and *H. pylori*

The observed incidences of glandular stomach adenocarcinomas are shown in Table 2. Glandular stomach adenocarcinomas, histologically featuring tubular structures with cellular atypia infiltrating into the muscle layer, were found in eight animals treated with both NIAN and *H. pylori* (8/26 = 31%) at 54–104 weeks. All adenocarcinomas were observed in the pyloric mucosa and located in the lesser curvature of the stomach, where macroscopically severe edematous thickening was also seen (Fig. 4*a*). The observed adenocarcinomas in seven animals were of well differentiated (Fig. 4*b*), and a moderately differentiated lesion was observed in one animal (Fig. 4*c*). In the animals treated with broth alone, broth + NIAN and *H. pylori* alone (groups A, B and C), no glandular stomach adenocarcinomas were observed. The incidence of glandular stomach adenocarcinomas in group D was significantly higher than that in groups A, B and C ($p < 0.05$, $p < 0.01$ and $p < 0.05$, respectively).

Irrespective of NIAN treatment and *H. pylori* infection, skin tumors, which histologically were well to poor differentiated squamous cell carcinomas, sebaceous carcinomas and melanomas, were found in one animal (1/15 = 7%) in group A, three animals (3/22 = 14%) in group B, two animals (2/18 = 11%) in group C and five animals (5/26 = 19%) in group D. A hemangioma was also observed in a kidney of one animal in group D (1/26 = 4%). No significant differences were apparent in these tumor incidences among groups A–D.

Discussion

In the present study, NIAN was found to induce glandular stomach adenocarcinomas in MGs in combination with *H. pylori* infection. NIAN-DNA adducts were also detected in the glandular stomach of MGs after treatment with NIAN, although clarification of their chemical structure(s) has yet to be performed. DNA adducts observed in the glandular stomachs of NIAN-treated MGs probably contain an indole-3-acetonitrile moiety. However, it is further likely that NIAN would act as an NO donor under aqueous conditions, thereby causing DNA modifications.[31–33] In fact, Lucas *et al.* demonstrated that NIAN can efficiently transfer nitroso groups to nucleophilic targets in purine nucleotides, causing *N*-nitrosation, deamination and the formation of a novel guanine analog, oxanine.[33]

Glandular stomach adenocarcinomas induced by NIAN treatment plus *H. pylori* infection were located in the pyloric region, similar to MNNG or MNU treatment plus *H. pylori* infection-induced glandular stomach adenocarcinomas in MGs.[26,27] Meanwhile, no glandular stomach cancers were observed in the groups of *H. pylori*-infected MGs without NIAN treatment, which is consistent with previous studies,[26,27] nor in the group treated with only NIAN. These findings indicated that *H. pylori* is a strong promoter of gastric carcinogenesis. Histological examination revealed that the tumors developed by NIAN + *H. pylori* were of well or moderately differentiated adenocarcinomas. Well or poorly differentiated adenocarcinomas and signet ring cell carcinomas were observed in *H. pylori*-infected MGs treated with MNNG or MNU.[26,27] Further studies are required to clarify the histological variety of stomach adenocarcinomas induced by NIAN, MNNG or MNU, since the type of cancer might depend on the genotoxic action of chemical carcinogens, rather than the effects of *H. pylori* infection.[27] In addition, tumors were observed in skin and kidney, which were suspected to spontaneously develop. The MGs have been reported to develop spontaneous skin tumors such as sebaceous and squamous cell carcinoma.[34]

Epidemiological studies have indicated that nitrate intake increases gastric cancer risk, and major sources are vegetables including Chinese cabbage, spinach and parsley.[14] Indole-3-acetonitrile, a precursor of NIAN, is distributed widely in cruciferous vegetables including Chinese cabbage and sprouts.[35] Furthermore, fava beans (*Vicia faba*), which are commonly consumed in Colombia, give rise to a potent mutagen in the presence of nitrite under acidic conditions.[36] The nitrosatable precursor of the mutagen in fava beans and the major product of nitrosation are reported to be an indole compound, 4-chloro-6-methoxyindole and an *N*-nitroso compound, 4-chloro-2-hydroxy-N^1-nitroso-indolin-3-one oxime, respectively.[37] Other indole compounds are also reported to produce direct-acting mutagens after nitrite treatment under acidic conditions.[38,39] In general, conversion of indole derivatives to nitrosated forms *in vitro* is known to be rapid and efficient at physiologically feasible nitrite concentrations with the low pH of the human stomach.[37] Thus, it is conceivable that nitrosation of indole compounds such as indole-3-acetonitrile probably occurs in human stomach. On the other hand, nitric oxide is suggested to be produced by activated macrophages in inflamed organs with *H. pylori* infection.[18] Therefore, nitrosation of indole compounds could be mediated by both acid catalysis and inflammatory responses in the human stomach.[18,20,37–40] On the basis of the conversion rate of NIAN from indole-3-acetonitrile under physiological conditions, the dose of NIAN used in the present study appears about 500–1000 fold the expected human exposure to NIAN *via* fresh or pickled Chinese cabbage. However, humans continually consume various kinds of foods containing indole compounds and nitrate during ordinary life. Thus, it is probable that the total amount of nitroso-indole compounds would be much closer to the dose of NIAN used in the present study. Moreover, it has been reported that low doses of chemical carcinogens, such as MNNG and MNU, could induce glandular stomach cancers in rodents under inflammation conditions including NaCl treatment and *H. pylori* infection, but hardly induce glandular stomach cancer without NaCl treatment and *H. pylori* infection. Therefore, the continuous intake of indole compounds and nitrate may play an important role for gastric carcinogenesis in East Asian countries still with a high salt consumption and *H. pylori* infection rate.

Gastric cancer is tending to decline in most countries.[41–43] One of the explanations for this tendency is the reduced prevalence of *H. pylori* infection.[42] Changes in dietary habits, mainly being lower salt consumption, could be also related to reduced gastric cancer incidence. However, the gastric cancer prevalence in East Asian countries, such as Japan and Korea, is still high.[2] At present, we have not succeeded in detecting NIAN in human bodies nor the exposure levels of the precursor, indole compounds for humans. Thus, it is necessary to estimate the human exposure levels to nitroso-indole compounds including NIAN, and to study further animal experiments and epidemiological analyses for clarification of contribution of nitroso-indole compounds under *H. pylori* infection in humans gastric carcinogenesis.

In conclusion, the present study demonstrated that NIAN can induce gastric cancer in *H. pylori*-infected MGs. It is noteworthy that nitrosatable precursors widely exist in foods. Thus, it is suggested that *N*-nitroso indole compounds including NIAN might contribute to the frequent development of gastric cancer in East Asian countries such as Japan and Korea in which the prevalence of *H. pylori* infection is relatively high. Further studies of interaction with other dietary elements appear warranted to promote the prevention of human gastric cancer.

Acknowledgements

The authors thank Dr. Nobuo Takasuka, Naoaki Uchiya and Yusaku Hori for their expert technical assistance. S.T. is presently the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

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DNA Repair 11 (2012) 259–266

Contents lists available at SciVerse ScienceDirect

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Activation of AMP-activated protein kinase by MAPO1 and FLCN induces apoptosis triggered by alkylated base mismatch in DNA

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ARTICLE INFO

Article history:
Received 31 August 2011
Received in revised form 9 November 2011
Accepted 28 November 2011
Available online 29 December 2011

Keywords:
AMPK
Apoptosis
Folliculin/BHD
MAPO1/FNIP2/FNIPL
O^6-methylguanine

ABSTRACT

O^6-Methylguanine produced in DNA by the action of simple alkylating agents, such as *N*-methyl-*N*-nitrosourea (MNU), causes base-mispairing during DNA replication, thus leading to mutations and cancer. To prevent such outcomes, the cells carrying O^6-methylguanine undergo apoptosis in a mismatch repair protein-dependent manner. We previously identified MAPO1 as one of the components required for the induction of apoptosis triggered by O^6-methylguanine. MAPO1, also known as FNIP2 and FNIPL, forms a complex with AMP-activated protein kinase (AMPK) and folliculin (FLCN), which is encoded by the *BHD* tumor suppressor gene. We describe here the involvement of the AMPK–MAPO1–FLCN complex in the signaling pathway of apoptosis induced by O^6-methylguanine. By the introduction of siRNAs specific for these genes, the transition of cells to a population with sub-G_1 DNA content following MNU treatment was significantly suppressed. After MNU exposure, phosphorylation of AMPKα occurred in an MLH1-dependent manner, and this activation of AMPK was not observed in cells in which the expression of either the *Mapo1* or the *Flcn* gene was downregulated. When cells were treated with AICA-ribose (AICAR), a specific activator of AMPK, activation of AMPK was also observed in a MAPO1- and FLCN-dependent manner, thus leading to cell death which was accompanied by the depolarization of the mitochondrial membrane, a hallmark of the apoptosis induction. It is therefore likely that MAPO1, in its association with FLCN, may regulate the activation of AMPK to control the induction of apoptosis triggered by O^6-methylguanine.

1. Introduction

Most of the DNA lesions produced by internal and external agents can be removed by cellular DNA repair enzymes, while cells with un-repaired lesions are eliminated by apoptosis. The biological significance of these two mechanisms is clearly shown when organisms lacking one or both of these cellular functions are exposed to simple alkylating agents, such as *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which alkylate purine and pyrimidine bases in DNA [1]. Among the various modified bases thus produced, O^6-methylguanine is of particular importance since this modified base can pair with thymine as well as cytosine during DNA replication, leading to induction of mutation and cancer [2,3]. Organisms possess a specific DNA repair enzyme, O^6-methylguanine–DNA methyltransferase (MGMT), which transfers a methyl-group from O^6-methylguanine in DNA onto the enzyme molecule, thereby repairing the DNA lesion in a single step reaction [4,5]. When the modified base is not repaired, an O^6-methylguanine–thymine pair is formed through DNA replication and this mismatch can be recognized by a mismatch repair protein complex, composed of MSH2, MSH6, MLH1 and PMS2, which induces apoptosis to exclude cells carrying the mutation-evoking DNA lesions [6–8]. It is noteworthy that *Mgmt*$^{-/-}$ mice, which lack the DNA repair enzyme specific for O^6-methylguanine, are hypersensitive to both the killing and to the tumorigenic action of alkylating chemicals [9–12] and these dual effects can be dissociated by the introduction of an additional defect in mismatch repair genes. Mice with mutations in both alleles of the *Mgmt* and the *Mlh1* genes, the latter encoding a protein involved in the recognition of mismatched base, are as resistant to MNU as are wild-type mice in terms of survival, but are much more susceptible to MNU-induced tumorigenesis than wild-type mice

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doi:10.1016/j.dnarep.2011.11.006

[13]. Consistent with these results, *Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$ cells, derived from the gene-targeted mice, are unable to induce apoptosis and show an elevated mutant frequency after MNU treatment [14].

The apoptotic signal initiated through the mismatch recognition complex activates a signaling cascade leading to the cell cycle checkpoints and apoptotic pathways for cell death. Both the release of cytochrome C from the mitochondria as well as the activation of Apaf-1 and caspase-3, hallmarks of the induction of apoptosis, have been demonstrated after the treatment of cells with alkylating agents that produce O^6-methylguanine [14,15]. However, the precise molecular mechanism underlying the signal transduction downstream of mismatch recognition still remains to be determined. To identify the factors involved in the O^6-methylguanine-induced apoptotic process, we screened MNU-resistant clones derived from MNU-sensitive *Mgmt*$^{-/-}$ cells using retrovirus-mediated gene-trap mutagenesis [16]. Mouse-derived KH101 cells, carrying an insertional mutation in one of the alleles of an uncharacterized gene, were unable to induce mitochondrial membrane depolarization as well as caspase-3 activation, after treatment with MNU. In this way, we identified a new gene, designated as *Mapo1* (O^6-methylguanine induced apoptosis 1), which was related to the induction of apoptosis. The mutant frequency of KH101 cells was significantly elevated after the treatment with MNU, thus supporting the notion that the induction of apoptosis, in which the MAPO1 is involved, contributes significantly to the elimination of cells carrying mutation-inducing DNA lesions. A search in the database revealed that the amino acid sequence of the MAPO1 protein is homologous to that of folliculin-interacting protein 1 (FNIP1), which was identified as a protein having the capacity to associate with folliculin [17]. Folliculin is a tumor suppressor protein with unknown biological activity, and is encoded by the *FLCN* gene. Mutations in the *FLCN* gene have been found in patients with Birt-Hogg-Dubé (BHD) syndrome [18,19], which is characterized by the development of hair follicle hamartomas, lung cysts, and an increased risk for renal neoplasia [20–22]. Identification of another folliculin-interacting protein, displaying a similarity in its amino acid sequence to that of FNIP1, was reported by two groups of researchers and the gene responsible was named *FNIP2* and *FNIPL*, respectively [23,24]. The *FNIP2/FNIPL* gene turned out to be the same gene as the human homolog of *Mapo1*. It was also reported that FNIP2/FNIPL, as well as FNIP1, could bind to 5′-AMP-activated protein kinase (AMPK), composed of AMPKα, β and γ subunits, which is an important energy sensor in cells that negatively regulates cell growth and proliferation [25,26].

We report here that a complex composed of MAPO1, FLCN and AMPK is involved in the induction of apoptosis triggered by O^6-methylguanine–thymine mispair. Evidence is presented which shows that during the course of apoptosis induction, the phosphorylation of AMPKα occurs in a MAPO1- and FLCN-dependent manner.

2. Materials and methods

2.1. Cell lines and cell culture

The YT102 (*Mgmt*$^{-/-}$ *Mlh1*$^{+/+}$), YT103 (*Mgmt*$^{-/-}$ *Mlh1*$^{-/-}$) and KH101 (*Mgmt*$^{-/-}$ *Mapo1*$^{+/-}$) cell lines were established as described previously [14,16]. The cells were cultivated in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2.

2.2. Chemicals

N-Methyl-*N*-nitrosourea (MNU) was obtained from Sigma. Compound C and AICA-Ribose were purchased from Calbiochem.

2.3. Immunoprecipitation and immunoblotting

To prepare cells expressing Flag-tagged MAPO1 or HA-tagged FLCN, a pIRES-puro3 vector (Clontech) containing mouse-derived *Mapo1* cDNA tagged with Flag epitope at the carboxy terminal end or a pIRES-puro2 (Clontech) vector carrying mouse-derived *Flcn* cDNA tagged with the HA epitope at the amino terminal end was introduced into YT102 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the immunoprecipitation, the cells were lysed with NETN buffer (50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.2% NP-40, 1 mM EDTA) containing protease inhibitors (Roche). To precipitate the Flag-tagged MAPO1, 10 μl of anti-FLAG M2-agarose (Sigma) were added to the extract, and incubated for 4 h at 4 °C. Alternatively, 10 μl of anti-HA (HA-7)-agarose (Sigma) were added to precipitate the HA-tagged FLCN, and the mixture was incubated overnight at 4 °C. After extensive washing of the beads with NETN buffer, the proteins bound to the beads were eluted in 40 μl of 2× SDS-PAGE sample buffer (120 mM Tris/HCl (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT, 0.002% bromophenol blue).

For the immunoblotting analyses, immunoprecipitated materials or whole cell extracts prepared by the lysis of cells with 2× SDS-PAGE sample buffer were subjected to SDS-PAGE and electroblotted onto a PVDF membrane (Bio-Rad). Detection was performed using an ECL Plus or Advance Western blotting detection kit (GE Healthcare). The primary antibodies used were: anti-FLAG M2 (Sigma), anti-HA HA-7 (Sigma), anti-FLCN (Protein Tech Group, Inc.), anti-AMPKα (Cell signaling), anti-β-actin (Sigma), and anti-phospho-AMPKα (Thr172) (Cell signaling). Anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare) were used as the secondary antibodies.

2.4. siRNA transfection

Stealth RNAi for the *Mapo1* gene (siMapo1), 5′-CAGAAAGCAGAGGAUGUUCCUAUUA-3′, *Flcn* gene (siFlcn#1), 5′-UUAUUCAGGAUAGUGGGCCCAACUC-3′, (siFlcn#2), 5′-UGGUGACUGACGUACUUAAUAGAGG-3′, and *Ampkα* gene (siAmpkα#1), 5′-UAUCUUAGCGUUCAUCUGGGCAUCC-3′, (siAmpkα#2), 5′-AAGAUGAUAAGCCACUGCAAGCUGG-3′ were purchased from Invitrogen. After culturing 1×10^5 cells in a 6-well plate for one day, the cells were transfected with 20 nM siRNA, using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. For the control transfection, Stealth RNAi Negative Control Medium GC Duplex (Invitrogen) was used.

2.5. Flow cytometric analysis

For the sub-G_1 population assay, cells were washed with PBS and suspended in 400 μl of PBS containing 0.1% Triton X-100, 25 μg/ml of propidium iodide and 0.1 mg/ml of RNase A. The samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson), with 10,000 events per determination.

For the mitochondrial membrane depolarization assay, cells were treated with the MitoProbeTM DiOC2(3) Assay Kit (Invitrogen), according to the manufacturer's protocol, and then subjected to analysis using a FACS Calibur flow cytometer.

2.6. Trypan blue exclusion assay

The viability of YT102, KH101 and siRNA-transfected YT102 cells was assayed, based on their trypan blue exclusion. The cells treated with AICA-Ribose were collected 48 h after the drug treatment and were stained with 0.2% trypan blue. The percentage of dead cells was determined as the percentage of trypan blue staining-positive cells. At least 500 cells were counted per experiment.