Tumor Induction by Azoxymethane (AOM) and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in F344 Rat Gastric Mucosa Featuring Intestinal Metaplasia Caused by X-irradiation

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Male F344 5-week-old rats were X-irradiated, and 16 weeks after the first dose, azoxymethane (AOM) was injected or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) was given by intragastric intubation. Tumors in the pylorus of the glandular stomach were observed in 4 out of the 29 animals receiving X-rays + AOM and in 4 out of the 25 animals receiving X-rays + PhIP, 12 months after administration. No such lesions were found in the chemical or X-ray alone groups. Intestinal metaplasia and some induced tumors were positive for CDX2. It was concluded that the presence of intestinal metaplasia may increase sensitivity to the induction of gastric tumors by colon carcinogens.

Key Words: Gastric tumor, Azoxymethane, 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, F344 rats, Intestinal metaplasia

Based on investigations in humans, intestinal metaplastic changes in the stomach have been considered precancerous lesions or a predisposing condition for differentiated gastric carcinoma development (1-7). However, we experimentally investigated an inverse relationship between quantity of intestinal metaplasia, with or without Paneth cells, and gastric tumor development, and established that its presence does not exert a positive influence on induction of gastric neoplasia by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methylnitrosoure (MNU) in rats (8,9). The situation is complex, however, because Nakagawa et al. have indicated that colorectal mucosa implanted into the glandular stomach, like the intrinsic large intestine, is sensitive to tumorigenesis caused by the colon carcinogen, 1,2-dimethylhydrazine (DMH), in contrast to normal gastric mucosa (10). Furthermore, we reported that induction of intestinal metaplastic mucosa in the glandular stomach is associated with susceptibility to tumorigenesis due to DMH (11,12).

The present study was designed to further examine whether intestinal metaplasia might be a target for azoxymethane (AOM) or 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)-induction of malignant tumors in the glandular stomach.

Materials and Methods

Animals. Male F344/DuCrj rats, 5 weeks of age at the commencement, were purchased from Charles River and housed five to a polycarbonate cage under constant conditions of temperature (24 ± 2°C) and relative humidity (55 ± 10%), with a 12:12-hour light-dark cycle. The animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. All rats were provided with a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

The animals were X-irradiated according to the method described previously (8,9,13), with two X-ray doses of 10 Gy each at a three-day interval (total dose, 20 Gy).

Four months after the first irradiation, initiation of azoxymethane (AOM, Sigma Chemical Co., St. Louis, MO) (14,15) or 2-amino-1-methyl-6-pheny-

limidazo [4,5-b]pyridine (PhIP, NARD, Amagasaki, Japan), both of which induce colon cancer (16-19), was commenced. AOM was given in weekly subcutaneous injections of 15 mg/kg body wt for 3 weeks, while PhIP was administered every 2 days, 3 times per week for a total of 10 doses of 75 mg/kg body wt by intragastric intubation.

Experimental Procedure. A total of 169 rats was divided into 6 groups. The animals in Groups 1 to 3 were X-ray irradiated. Those in Groups 1 and 4 were given PhIP, while Groups 2 and 5 received AOM. All were fed a normal MF diet throughout the experimental period. The animals were killed and autopsied when they became moribund and all remaining rats were killed by ether anesthesia 12 months after the initial chemical carcinogen treatment. The stomach, and the small and large intestinal tracts were removed, opened and extended on cardboard for inspection. The location of individual tumors was recorded by measuring the distance from the pyloric ring in the small intestine and from the anus in the large intestine. The numbers and sizes of individual tumors were also noted. Whole tissues were fixed in 10% neutral formalin. Alkaline phosphatase (ALP)-positive foci in the gastric mucosa were detected by the naphthol-AS-MX-phosphate-fast blue RR staining method (20) and the numbers of ALP-positive foci in the whole gastric mucosa per rat were counted under a dissection microscope with a double-blind protocol. Sections of paraffin-embedded tissue were routinely stained with hematoxylin and eosin, and for clarification, when necessary, with periodic acid Schiff-Alcian-blue (AB-PAS). Other organs were removed, fixed in 10% neutral formalin and stained with HE.

Intestinal metaplasias were categorized using the following histological criteria (21,22): type A, gastric mucosa with goblet cells which were positive for AB-PAS; type B, intestinal-type crypts without Paneth cells or type C, intestinal metaplasia with Paneth cells (alkaline phosphatase-positive foci). Using these criteria, the numbers of metaplastic crypts were counted separately for 2 sections through the lesser curvature (pylorus) and 4 through the greater curvature (fundus) in a double-blind fashion. Tumors in the stomach, small intestine and large intestine were classified into two types, adenoma, especially stomach, atypical hyperplasia (ATP, shown in Fig.1) and adenocarcinomas invading the musclaris mucosa or further, and also into two histological types, the well-differentiated (Fig.2) and poorly-differentiated types (Fig.3), the latter including both mucinous and signet ring cell forms.

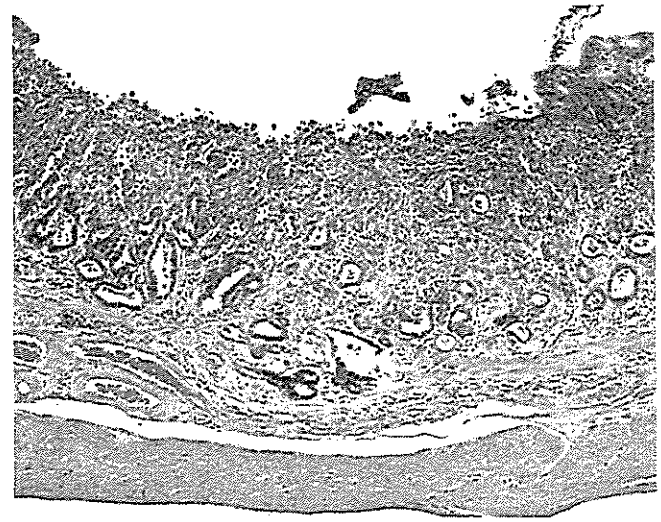

Fig. 1 - Atypical hyperplasia shown proliferation of atypical glands in mucosa, x100, HE staining.

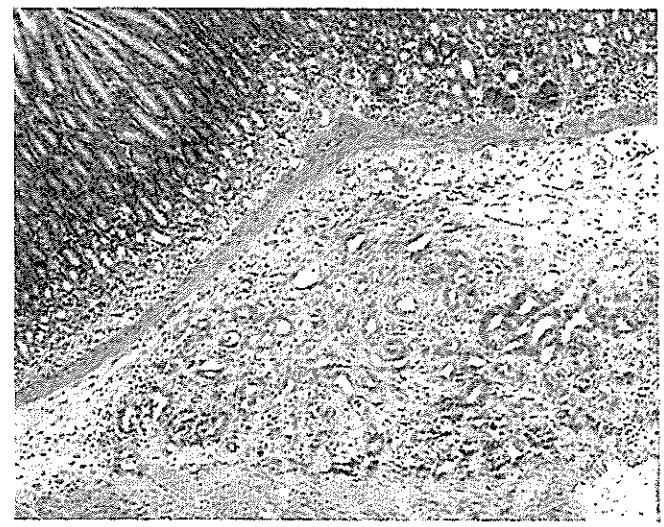

Fig. 2 - Well differentiated adenocarcinoma shown atypical glands invaded all the layer of the gastric wall, x100, HE staining.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three-μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against CDX2 (diluted 1:50; Biogenex CDX2-88) (23) for 1 hour at

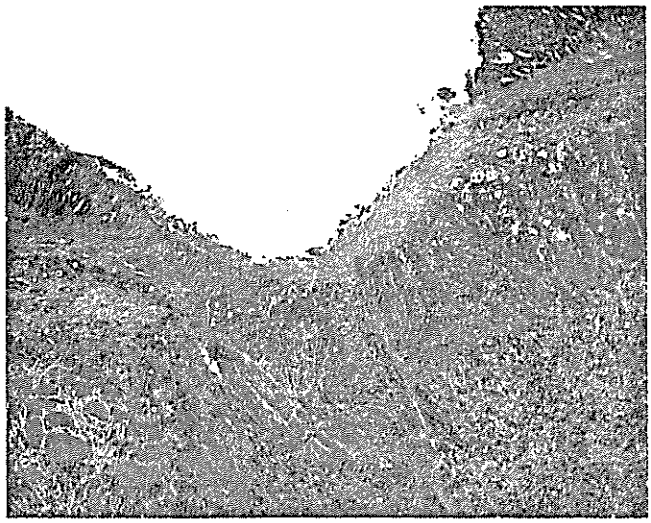

Fig. 3 - Signet ring carcinoma, x40, HE staining.

room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca., Catalog No. PK-8800) and peroxidase-conjugated streptavidin complexes. Peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. CDX2-positive cells were observed.

Statistical analysis. The significance of differences in numerical data was evaluated by using the chi-squared test and Student's t test.

Results

Mean survival did not significantly differ among the groups. Body weights in the chemical carcinogen treatment groups were significantly decreased as compared to those in the control group (Table I). Heart weights were lower in the X-ray + PhIP and X-ray and PhIP groups and liver in the AOM, kidneys in the PhIP and AOM, testes in the AOM and spleens in the X-ray + PhIP and X-ray groups were significantly smaller than those of the controls. On the other hand, spleen weights significantly increased. Relative liver, kidney, and testis weights (relative weight; organ weight/body weight x 1,000) in the X-ray + PhIP group and relative spleen weights in the AOM group were significantly enlarged, while in the X-ray goup they were smaller than those in the controls (Table II).

The incidence and number of areas of intestinal metaplasias in the X-ray irradiated groups were significantly increased as compared to those in the non-irradiated groups (Tables III and IV). Incidences in

Table I - Body and organ weights

Group	Mean survival	Body Weight (g)	Organ weight (g)					
			Heart	Liver	Kidney	Adrenal	Testis	Spleen
X-ray+PhIP	356±32	386±50**	1.12±0.09**	11.6±1.5	2.47±0.35	0.052±0.006	3.24±0.18	0.60±0.13**
X-ray+AOM	337±50	415±37**	1.19±0.11	12.0±3.0	2.36±0.21	0.084±0.101	3.36±0.34	0.80±0.42
X-ray	365±11	433±35	1.14±0.13**	11.7±1.1	2.37±0.19	0.056±0.014	3.35±0.27	0.63±0.13**
PhIP	364	425±26**	1.14±0.10**	11.3±1.0	2.29±0.23*	0.063±0.012	3.21±0.30	0.79±0.10
AOM	359±10	427±23*	1.21±0.11	11.2±1.1*	2.29±0.14*	0.055±0.010	3.14±0.35**	1.09±0.19**
Control	364	452±33	1.26±0.12	12.2±1.3	2.43±0.20	0.065±0.015	3.39±0.29	0.86±0.10

**: Significantly difference from Control value (P<0.05) - **: Significantly difference from Control value (P<0.01)*

Table II - Relative weight*

Group	Heart	Liver	Kidney	Adrenal	Testis	Spleen
X-ray+PhIP	2.96±0.59	30.7±5.9**	6.56±1.52**	0.139±0.031	8.61±1.92**	1.59±0.43
X-ray+AOM	2.88±0.26	29.1±8.2	5.69±0.38	0.199±0.226	8.16±1.04	1.96±1.14
X-ray	2.66±0.33	27.1±2.4	5.49±0.43	0.131±0.037	7.76±0.61	1.47±0.42**
PhIP	2.68±0.18	26.7±1.9	5.41±0.72	0.149±0.031	7.58±0.76	1.88±0.30
AOM	2.83±0.23	26.1±1.8	5.37±0.31	0.128±0.021	7.35±0.84	2.55±0.46**
Control	2.78±0.24	26.9±1.7	5.39±0.37	0.144±0.031	7.52±0.68	1.91±0.20

**:Organ weight/Body weight x 1,000 - **: Significantly difference from Control value (P<0.01).*

Table III - Incidence of Intestinal metaplasia (%)

Group	ALP	Pylorus				Pylorus+Fundus			
		A	B	C	Total	A	B	C	Total
X-ray+PhIP	81	32	91	59	95	36	95	50	95
X-ray+AOM	66	26	78	65	82	26	87	74	87
X-ray	66	0	94	47	94	0	94	50	94
PhIP	0	8	0	0	8	8	8	0	25
AOM	0	17	8	0	25	17	25	0	33
Control	0	0	0	0	0	0	11	0	11

ALP: Alkaline phosphatase positive intestinal metaolasia - A: Goblet cells with the gastric musosa
B: Intestinal type crypt without Paneth cells - C: Intestinal type crypt with Paneth cells.

the X-ray groups were 84-95%, and in the non-irradiated groups were only 11-33%. The number of type B metaplasias and totals in the X-ray groups were significantly increased, and type C lesions were also more common in the X-ray + AOM and X-ray alone groups than in the controls (Table IV).

The first tumor appeared at 204 days, in an X-ray + AOM animal. Total tumors in the AOM groups were significantly more numerous than in the PhIP groups. Gastric tumors in the glandular stomach were observed in four out of the 25 (17%, three ATP, one adenocarcinoma) X-ray + PhIP animals, and four out of the 29 (10%, one ATP and three adenocarcinomas) X-rays + AOM animals. One signet ring cell carcinoma was found in this group. All other tumors in the glandular stomach were well-differentiated without goblet cells or mucin and were located in the middle or upper portion. Nuclei of

Table IV - Mean number of intestinal metaplasia

Group	ALP	Pylorus				Pylorus+Fundus			
Group		A	B	C	Total	A	B	C	Total
X-ray+PhIP	19.1±26.4	0.6±1.1	6.9±5.5*	1.4±1.8	8.9±7.3*	0.6±1.1	7.4±6.2*	1.5±1.9	9.5±8.2**
X-ray+AOM	25.0±42.2*	0.4±0.8	8.4±7.7**	2.0±2.2*	10.9±9.3**	0.4±0.8	9.1±7.5**	2.4±2.2*	11.9±8.7**
X-ray	11.4±18.9	0	9.8±10.0**	1.8±3.4	11.2±11.4**	0	10.7±10.9**	2.3±3.7*	12.7±12.6**
PhIP	0	0.1±0.3	0.8±2.6	0	0.8±2.6	0.1±03	1.1±2.8	0	1.2±2.8
AOM	0	0.5±1.4	0.2±0.6	0	0.7±0.6	0.5±1.4	0.4±0.8	0	0.9±1.5
Control	0	0	0	0	0	0	0.1±0.3	0	0.1±0.3

**: Significantly difference from Control value (P<0.05) - **: Significantly difference from Control value (P<0.01)*

intestinal metaplasia (Fig.4), and the signet ring cell carcinoma (Fig.5) were positive for CDX2 by immunohistochemistry. On the other hand, the cytoplasm of a well-differentiated adenocarcinoma was positive (Fig.6). No gastric tumors were observed in the other groups (Table V).

The incidence of small intestinal tumors was 28% and 3% in the X-ray+AOM and AOM groups, respectively. The more frequent colon tumors were the multiple and the papillary and polypoid types. The incidence of colon tumors was 8%, 12%, 79% and 72% in the X-ray + PhIP, PhIP, X-ray + AOM and AOM groups, respectively, and the number of tumors was 0.08 ± 0.28, 0.12 ± 0.3, 1.31 ± 0.97 and 1.17 ± 1.04, respectively (Table V). The incidences of signet ring cell carcinomas were 2 (7%) in the X-ray + AOM and 5 (19%) in the AOM groups. Aberrant crypt foci were observed in all of the chemical carcinogen-treated groups (data not shown).

Pancreas (17-38%) and skin tumors (6-28%) also developed after X-ray treatments. Three kidney tumors were found in the X-ray + PhIP group, three

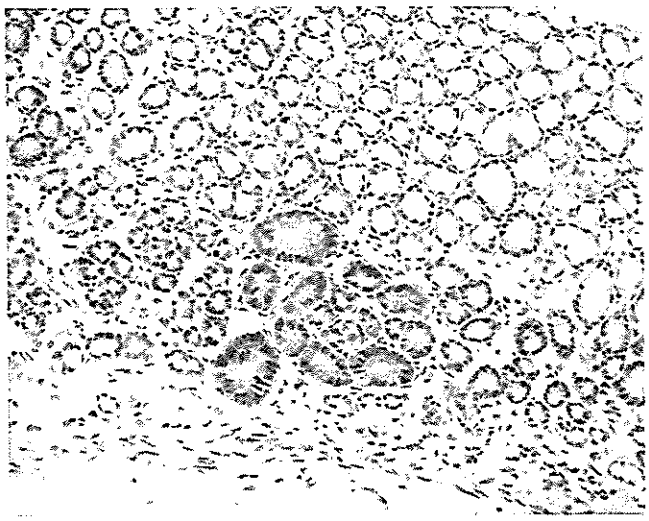

Fig. 4 - CDX-2 positive nuclei were observed in glands of intestinal metaplasia, x100, CDX-2 antibody staining.

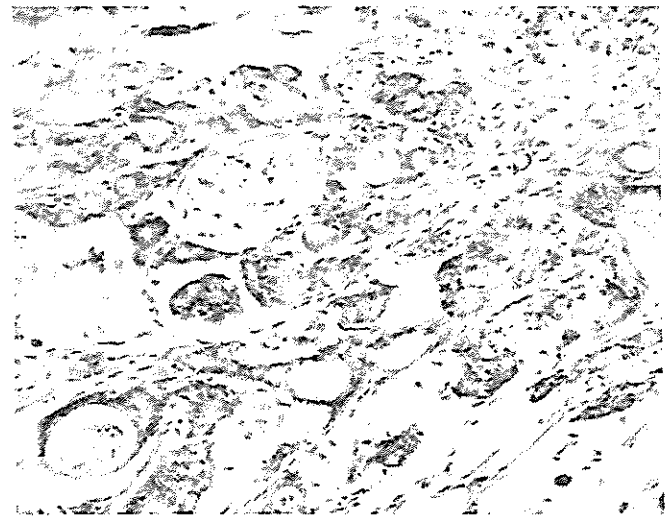

Fig. 5 - CDX-2 positive nuclei were observed in signet ring cell carcinoma, x 200, CDX-2 antibody staining.

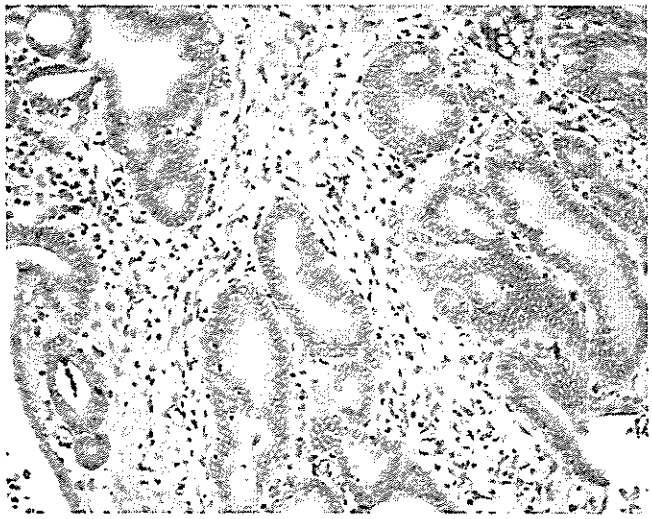

Fig. 6 - CDX-2 positive cytoplasm was observed in well differentiated adenocarcinoma x 200, CDX-2 antibody staining.

ear duct tumors, one bone and two liver tumors in the X-ray + AOM group, and one tail papilloma in the PhIP group (Table V).

Discussion

In the present experiment, induction of intestinal metaplastic mucosa in the glandular stomach was associated with susceptibility to tumorigenesis induced by PhIP and AOM, in contrast to non-susceptible normal gastric mucosa. In earlier studies, regression analysis of gastric tumors per rat against the frequency of intestinal metaplasia, with or without Paneth cells, yielded a significant inverse relationship, suggesting that the development of intestinal metaplasia and gastric tumors might be independent (8,9). Previously, we reported that intestinal metaplasia is not susceptible to gastric tumor induction by MNNG and MNU (8,9) and that colonic mucosa transplanted into gastric mucosa lacks susceptibility to these carcinogens when given orally (24,25). Intestinal metaplasia or colorectal mucosa implants into the glandular stomach are sensitive to DMH carcinogenicity, whereas the normal gastric mucosa is not (10). Thus, it would appear that areas of intestinal metaplasia induced by X-irradiation might be susceptible to damage due to carcinogens targeting the large intestine. In the present experiment, CDX-2 appeared in intestinal metaplasia and in some of the gastric tumors. CDX-2 is not expressed in the normal stomach but is highly expressed in the normal intestine and intestinal metaplasia (23,27) and carcinoma of the stomach, indicating its involvement in these lesions. So, it is considered that some of the gastric tumors in this experiment might have been caused by intestinal metaplasia and/or circumstances

Table V - Incidence and number of colon tumors

	No	Total (%)	Gatsric (%)	Small intestine (%)	Colon tumor		Pancreas (%)	Skin (%)	Other (%)
					Incidence (%)	Number per rat			
X-ray+PhIP	25	16(64)	4(17)	0	2(8)	0.08±0.28	8(32)	7(28)	3(12) Kidney 3
X-ray+AOM	29	27(93)	4(10)	8(28)	23(79)	1.31±0.97	5(17)	3(10)	6(21) Ear duct 3 Liver 2 Bone 1
X-ray	32	15(47)	0	0	0	0	12(38)	2(6)	2(6) Squamous cell carcinoma 1 Papilloma 1
PhIP	25	4(16)	0	0	3(12)	0.12±0.33	0	0	1 Tail papilloma1
AOM	28	23(79)	0	1(3)	21(72)	1.17±1.04	0	0	2(9) Liver 1 Testis 1
Control	30	0	0	0	0	0	0	0	0

**:Significantly difference from X-ray group (P<0.05) - a: Significantly difference (P<0.05) - b: Significantly difference (P<0.01)*

of intestinal metaplasia.

We must consider the alternative possibility that the effects of irradiation and DMH and other colon carcinogens on glandular stomach epithelial cells are additive or synergistic. Tatemichi et al. reported that the cytochrome P450 monooxygenase 1A1 expressed in intestinal metaplasia, and carcinogen activation by 1A1 enzymes expressed in the gastric mucosa, may contribute to carcinogenesis of the stomach (28). It appears likely that intestinal mucosal stem cells are susceptible to colon carcinogenesis, independently of the administration route or their location. Thus, the intestinal mucosal phenotype appears to be the most important determinant of response to colon carcinogens, rather than the intestinal macro-environment itself.

In summary, the presence of intestinal metaplasia, with or without Paneth cells, may increase the sensitivity of the stomach to the induction of tumors by carcinogens like DMH, AOM or PhIP, but not by MNNG or MNU. The protocol used in the present experiment may provide a new approach for distinguishing between developmental events associated with intestinal metaplasia and gastric tumors.

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Protective effects of a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia and *Agaricus blazei* murill against X-irradiation in B6C3F1 mice: Increased small intestinal crypt survival and prolongation of average time to animal death

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Abstract. Radioprotective effects of a water-soluble extracts from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia (designed as MAK) and *Agaricus blazei* (Agaricus) against the shortening of survival time or the injury of crypt by X-irradiation were investigated in male B6C3F1 mice. MAK and Agaricus at three different doses were mixed into basal diet into biscuits at 5, 2.5 and 1.25% and administered from 1 week before irradiation. MAK (5% group) significantly prolonged animal survival as compared with basal diet group (control group) after 7 Gy of X-ray irradiation at a dose rate of 2 Gy min^{-1}. At doses of 8, 10 and 12 Gy X-irradiation at a dose rate of 4 Gy min^{-1} MAK (5% group) significantly increased crypt survival as compared to other groups. These results suggest that MAK can act as a radioprotective agent.

Introduction

One major goal of radiobiology research is the development of drugs that can be used to provide protection against radiation injury, and numerous compounds have been developed and tested. The observed protective effects point to the possibility of improving the therapeutic index of cancer radiotherapy, or reducing the acute radiation effects in persons exposed in accidents. The strategy of reducing radiation injury to normal tissues might thus have significant benefit in terms of medical applications. Hsu *et al* (1-4) reported radioprotective effects of several kinds of Chinese traditional prescriptions and enhanced immunocompetence after irradiation was found. These results have encouraged us to search for other drugs that might exert radioprotective influence.

Various mushrooms have a long history of use in folk medicine, and become subjects of great interest, due to their multiple nutritional and pharmacological properties. Mushroom extracts are widely sold as nutritional supplements and touted as beneficial for health. However, only a few studies are available on the biological effects of mushroom consumption. *Ganoderma lucidum* (Fr.) Karst, belonging to the Basidiomycetes class of fungi, is colloquially known as 'Rei-shi' or 'Mannentake' in China and Japan, and it has been attributed with various medical virtues handed down in folklore. *Ganoderma lucidum* exhibits anti-hepatoxic and free radical scavenging activity (5), exerts influence on the cell cycle and cellular signal transduction (6), inhibits leukemic-cell growth (7), and induces differentiation of leukemic cells into mature monocytes/macrophages (8). In addition, it may inhibit platelet aggregation (9), impede complex interactions of viruses with cell plasma membranes (10), inhibit tumor growth (11) and decrease the incidence of mouse lung tumors (12). A water soluble extract from cultured medium *Ganoderma lucidum* (Rei-shi) mycelia (designed MAK) contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. Previously, we have reported that MAK prevented the development of azoxymethane induced aberrant crypt foci (ACF.13), development of N,N'-dimethylhydrazine-induced colon tumors in ICR mice (14) and colon tumors induced by azoxymethane in F344 rats (15).

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Table I. Change of testis 28 days after 6 Gy X-irradiation.

Group	Size (μm)	Total no.	PCNA negative	Ratio (negative/total)
X-ray+ 5% MAK[a]	77.0±8.0	50.9±6.0	7.4±2.7[b]	14.0±5.0[c]
X-ray+ 5% Agaricus	70.0±8.0[c]	45.0±6.1	12.6±5.4[c]	28.0±11.0[c]
X-ray	75.0±8.0[c]	46.1±9.3	10.6±5.5	23.0±11.0
Control	175.0±14.0	29.0±3.8	0	0

[a]MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [b]$p<0.05$; [c]$p<0.01$.

The Basidiomycete mushroom *Agaricus blazei* Murrill, native to Brazil and popularly known in Japan as Himematsutake, has been largely produced and consumed as food and tea due to its medicinal effects, possibly including anti-carcinogenic activity (16,17). However, no experimental data exist regarding beneficial effects of this species of mushroom.

The present study was therefore conducted to assess the effects of MAK or *Agaricus blazei* extracts on crypt and animal survival after X-irradiation in mice.

Materials and methods

Animals. Six-week-old male B6C3F1 (Crj:B6C3F1) mice and our standard protocol for assessing radiation effects were employed in the present experiment. Animals were housed in polycarbonate cages, five per cage, and kept under constant conditions of temperature (24±2°C) and humidity (50±10%) with a 12 h light/12 h dark cycle, according to the Guide for Care and Use of Laboratory Animals established by Hiroshima University, and fed a commercial diet MF (Oriental Yeast Co. Ltd., Tokyo, Japan) alone or with a 5, 2.5 and 1.25% supplement of MAK and Agaricus in biscuits. Normal tap water was also provided *ad libitum*.

MAK and Agaricus. A water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) was prepared by Noda Shokkin-Kogyo Co., Ltd. (Chiba, Japan). In brief, *Ganoderma lucidum* (Rei-shi or Mannnentake) mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months, then the whole medium containing mycelia was extracted with hot water. The extract was filtered and spray-dried as MAK. Agaricus was purchased as a commercial powder of *Agaricus blazei* Murill.

Radiation. Groups of mice were whole body irradiated with 6 or 7 Gy of X-rays (each 10 animals) at a dose rate of 2 Gy/min for the animal survival study and 8, 10 or 12 Gy of X-rays once for crypt survival (each 5 animals) at a dose rate of

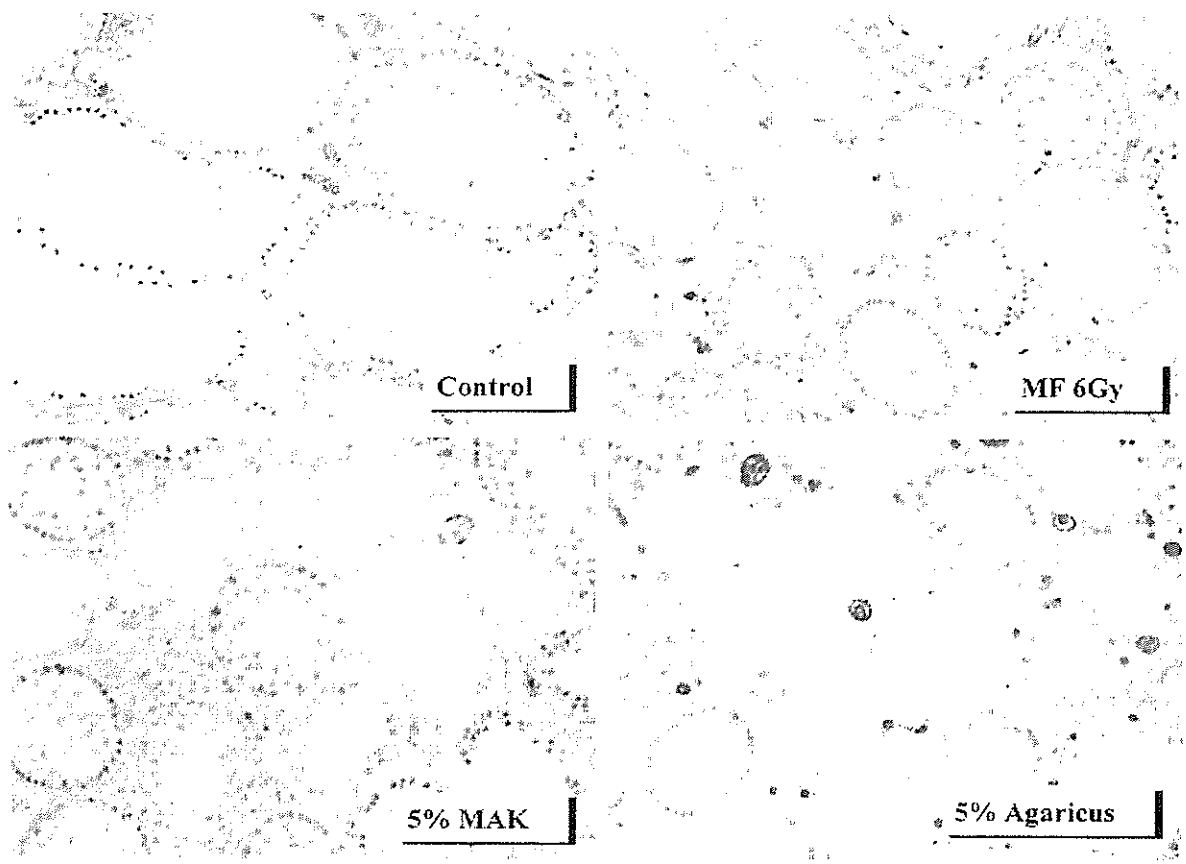

Figure 1. PCNA staining in seminiferous tubules.

Table II. Size of seminiferous tubules 4 weeks after 6 Gy whole body irradiation.

Group	Size (μm)
MF	75.0±7.8[a]
5% MAK	76.9±8.0
5% Agaricus	70.3±8.5[a]

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [a]Significantly different from 5% MAK.

4 Gy/min as measured with a Radocon 555 dosimeter. The mice were not anaesthetized during the irradiation. Exposure factors were as follows: 200 kVp and a half-value layer 1.18 mm Cu. The X-ray air dose (in R) was then converted to the absorbed dose (in cGy) using a factor of 0.95 cGy/R.

One week before irradiation, the mice were given a diet supplemented with MAK and Agaricus and kept for 28 days on the same diet after X-irradiation with 6 and 7 Gy. The animals were observed every day at 8:00, 12:00 and 18:00, and deaths were recorded for the animal survival experiment. In the other groups, the animals were kept for 3.5 days after irradiation then sacrificed for determination of crypt survival.

Autopsy. Immediately after sacrifice, segments of the jejunum from the ileocecal junction (30 to 40 cm) were removed and fixed in Carnoy's solution. They were cut into several pieces, bundled together, embedded in paraffin, sectioned at a thickness of 3 μm and stained with hematoxylin-eosin. To quantitative regenerating crypts, number of crypts per circumference was determined in cross-section (18). In each mouse the number of surviving crypts in 10 gut cross-sections was scored.

Animals were sacrificed after cumulative irradiation for 28 days. Testes were fixed in FSA solution (37% formalin 5 ml, 5% sucrose solution 15 ml and acetic acid 0.8 ml) for 5 days, then embedded, sectioned and stained routinely. Sizes of seminiferous tubules were measured. For immunohistochemistry, paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against monocolonal mouse anti-proliferating cell nuclear antigen antibody (Dako-PCNA. PC 10, code No. M 879) for 1 h at room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca, Catalog No. PK-8800) and peroxidase conjugated streptoavidin complexes. Peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. PCNA-positive cells in seminiferous tubules were counted.

Statistics. Statistical significance was determined with Dunnett's method and the Cox proportional hazard model for multiple comparisons using logarithmic transformation and the Student's t-test.

Results

Survival was not significantly affected with 6 Gy irradiation. Testes of surviving animals after 28 days of irradiation demonstrated significantly smaller seminiferous tubules in the 5% Agaricus group than with X-rays alone (Table I). The

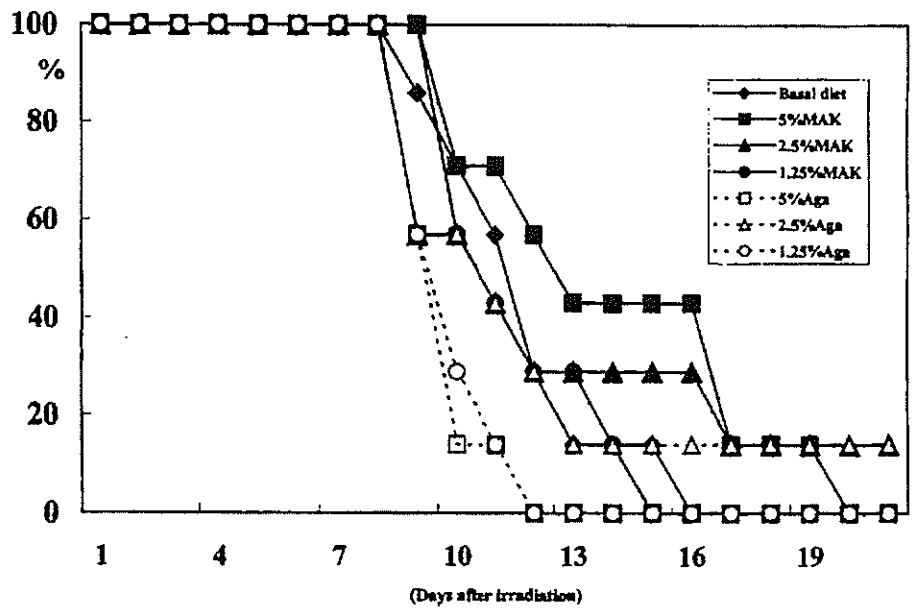

Figure 2. Survival after 7 Gy irradiation. MAK, a water-soluble extract at 5% from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. MAK vs. Basal diet $p<0.02$, 5% MAK vs. 1.25% MAK $p<0.02$, 5% MAK vs. 5% or 1.25% AGA $p<0.007$.

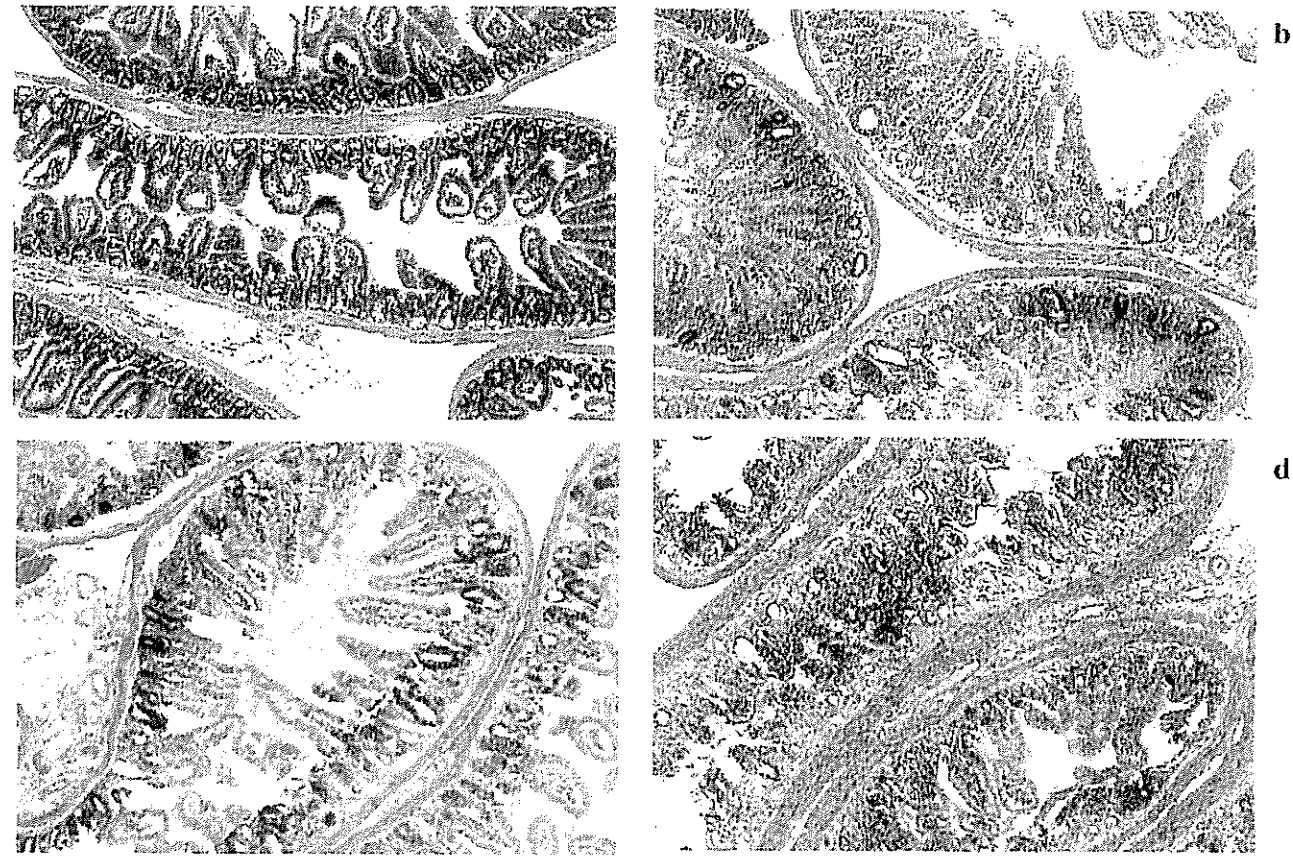

Figure 3. (a) Normal small intestine; (b) 10 Gy-irradiated small intestine in MF diet group. A few regenerated crypts were observed. (c) 10 Gy-irradiated small intestine in 5% MAK group. Many regenerated crypts were observed; (d) 10 Gy-irradiated small intestine in 5% Agaricus group. A few regenerated crypts were observed.

Table III. Crypt survival.

	0 Gy	8 Gy	10 Gy	12 Gy
MF	116.53±13.39	84.54±11.74	43.74±8.42	24.76±5.62
5% MAK		117.00±12.47	68.06±9.63	43.77±7.64
2.5% MAK		81.38±10.41	52.52±8.90*	28.08±5.49*
1.25% MAK		87.88±11.21	51.72±7.59*	29.78±4.44*
5% Agaricus		81.06±10.06	42.50±6.60	27.52±4.68*
2.5% Agaricus		83.52±10.18	49.98±7.30*	26.38±3.85
1.25% Agaricus		82.60±10.47	51.78±8.29*	27.02±4.98

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. 5% MAK was significantly different from other groups; ($p<0.01$). *Significantly different from MF group ($p<0.05$). *Significantly different from MF group ($p<0.01$).

number of PCNA-negative seminiferous tubules was zero in control animals (Fig. 1). Ration of PCNA negative vs. total seminiferous tubules in 5% MAK values was significantly smaller than that in 5% Agaricus values (Table II). Animals in Agaricus groups started to die 9 days after irradiation and survival is shown in Fig. 2. Delay in mortality was evident in 5% MAK group, with significantly increased survival in the MF ($p=0.02$), 1.25% MAK ($p=0.02$) and 1.25% Agaricus ($p=0.007$) by the Cox model.

The number of crypts in one circumference in the non-irradiated group was 116.5±13.4 (Fig. 3a). A dose-dependent decrease was evident with 8-12 Gy (Table III and Fig. 3b) and surviving crypts in 5% MAK (Fig. 3c) were significantly increased, compared to other groups in every dose. Crypt survival was evident with a significant difference in 2.5 and 1.25% MAK and Agaricus (Fig. 3d) ($p<0.01$) as compared with MF group in 10 Gy irradiation and in 2.5%, 1.25% MAK ($p<0.01$) and 5% Agaricus ($p<0.05$).

Discussion

The present paper documents a significant increase in the survival of crypts in animals receiving 5% MAK associated with a prolongation of average time to animal death after X-irradiation. Hsu *et al* (4,19) earlier reported that intraperioneal injections of the extract from *Ganoderma lucidum* before irradiation of 5 or 6.5 Gy X-rays improved the 30-day survival of ICR mice and increased recovery as assessed by hemograms, the 10-day blood forming stem cells (CFU) also being significantly higher for the *Ganoderma lucidum* treated group than for the untreated group. Chen *et al* (20,21) reported that administration of an extract of *Ganoderma lucidum* was able to enhance the recovery of cellular immunocompetence after 4 Gy-ray irradiation of ICR mice. It is well documented that radiation is a potent immunosuppressive agent, and moderate doses exert clear inhibitory effects on the counts of total leukocytes, lymphocytes and neutrophils. Radiation also has destructive effects on the leukocytepoietic organs such as the spleen, thymus and bone marrow, and protection effects of traditional Chinese medicines (1-4,19), ginseng (12,22,23) and garlic (24) have been investigated. They were able to enhance the recovery from decreased cellular immunocompetence, with protection or stimulation of the reticuloendothelial system, and induction of free radical scavenger. Recently, we reported that a water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) may stimulate the natural immune system or the aquire immune system in tumor-bearing mice (25). Therefore MAK might be a potent immunomodulater that up-regulates against immunosuppression by X-irradiation.

Houchen *et al* (26) have reported that expression of FGF-2 is induced by radiation injury and that recombinant human FGF-2 markedly enhanced crypt survival. Takahama *et al* (27) found that a replication-deficient adenovirus containing the HST-1 gene acts as a potent protector against lethal irradiation associated with injury to the intestinal tract as well as myelosuppression in the bone marrow and spleen. Farrell *et al* (28) have presented findings that recombinant human keratinocyte growth factor can protect mice from chemotherapy- and radiation-induced gastrointestinal injury and mortality, at least in terms of death from intestinal and marrow toxicity. We also found VEGF to have a protective influence (29). Cytokine-like substances in MAK may thus play an important role in the protection and/or the recovery and repopulation of critical tissue elements when given prior to and during radiation exposure. However, to our knowledge, there are no reports regarding cytokines in MAK.

MAK contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. *Ganoderma lucidum* mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months. Lignin is processed or converted to water-soluble lignin with enzymes during growth of mycelia. A water-soluble lignin is seemed to be the characteristic constituent, and does not contribute to every function of MAK, but is closely related to some function of MAK.

The present experiment provided evidence of recovery of seminiferous tubule size and DNA synthesis with MAK, but not Agaricus treatment. The testis is the most sensitive organ for radiation injury (30,31) and the turnover time from primary spermatogonia to sperm is 60 days (32). Thus, both the smaller testis size and number of PCNA-positive cells of seminiferous tubes in the Agaricus group as compared with the MAK group, are compelling evidence of less radiation protection.

Studies are now in progress in our laboratory to further elucidate the active compounds of the water-soluble extract, the mode of their action included immune parameter and further clinical study for pharmaceutical effects.

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Gene Trap Mutagenesis-based Forward Genetic Approach Reveals That the Tumor Suppressor OVCA1 Is a Component of the Biosynthetic Pathway of Diphthamide on Elongation Factor 2*

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OVCA1 is a tumor suppressor identified by positional cloning from chromosome 17p13.3, a hot spot for chromosomal aberration in breast and ovarian cancers. It has been shown that expression of OVCA1 is reduced in some tumors and that it regulates cell proliferation, embryonic development, and tumorigenesis. However, the biochemical function of OVCA1 has remained unknown. Recently, we isolated a novel mutant resistant to diphtheria toxin and *Pseudomonas* exotoxin A from the gene trap insertional mutants library of Chinese hamster ovary cells. In this mutant, the *Ovca1* gene was disrupted by gene trap mutagenesis, and this disruption well correlated with the toxin-resistant phenotype. We demonstrated direct evidence that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on elongation factor 2, the target of bacterial ADP-ribosylating toxins. A functional genetic approach utilizing the random gene trap mutants library of mammalian cells should become a useful strategy to identify the genes responsible for specific phenotypes.

OVCA1 is a tumor suppressor isolated from chromosome 17p13.3, a hot spot for chromosomal aberration in breast and ovarian cancers (1, 2). It has been shown that expression of OVCA1 is reduced in tumors and that exogenous expression of OVCA1 inhibited growth of ovarian cancer cells (3). Furthermore, a study using *Ovca1* gene knock-out mice clearly showed that OVCA1 regulates cell proliferation, embryonic development, and tumorigenesis (4). Even though the biological or cell biological functions have been elucidated, the biochemical function of OVCA1 has not been ascertained.

Diphthamide is a unique post-translationally modified histidine residue found only on translational elongation factor 2 (EF-2),[1] which catalyzes the translocation of peptidyl tRNA from the ribosome A site to the P site during peptide chain elongation. Diphthamide has been found in all eukaryote and archaebacteria, however not in eubacteria. The diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) inactivate EF-2 by ADP-ribosylating the diphthamide (5, 6). The biosynthesis of diphthamide is one of the most complex post-translational modifications, and by genetic complementation analyses it has been shown that five different genes in yeast (7) and at least three genes in CHO cells (8) are involved in diphthamide synthesis. To date, three genes responsible for diphthamide formation have been elucidated in yeast and human (9–11). The biochemical function of diphthamide as a target of bacterial ADP-ribosylating toxins has been well characterized; however, its physiological role in cells has remained to be clarified.

Bacterial toxins are useful and valuable tools for investigating cell functions; important knowledge concerning cell functions has been obtained by analyses of mutants of established cell lines. Indeed, much of knowledge concerning the mechanisms of toxicity of DT has been elucidated by the study of toxin-resistant mutants (7, 8, 12–18). Although many of the DT-resistant mutants have been isolated, to clarify the genes involved in DT sensitive and/or resistant phenotype was time consuming and sometimes difficult work, especially in mammalian cells.

To increase the efficiency of insertional mutagenesis in mammalian cells, retrovirus gene trap vectors have been developed (19, 20). Gene traps are based on the integration of a reporter gene lacking a promoter into the genome and its expression from a tagged endogenous promoter. When a gene trap vector integrates into expressed genes, insertional mutants can be easily selected by selectable phenotype conferred by the gene trap vector. It is possible to increase the proportion of cells with virus-induced mutations to two to three orders of magnitude higher than in cells containing unselected proviruses by retroviral gene trap selection (21). So far, mutants having a variety of phenotypes have been isolated from CHO cells. This is because CHO cells are hypodiploid and functionally hemizygous at a number of loci (22, 23). For that reason it was expected that a single gene trap insertional mutagenetic event might result in loss of gene functions in CHO cells.

To identify the obligate genes involved in DT sensitivity and/or resistance, including diphthamide biosynthesis, we screened mutants resistant to DT from a random gene trap insertional mutants library of CHO cells. Recently, we have been able to isolate a novel mutant resistant to DT and ETA in which a mutant *Ovca1* gene was disrupted by gene trap mu-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB194396.

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[1] The abbreviations used are: EF-2, elongation factor 2; DT, diphtheria toxin; ETA, *Pseudomonas* exotoxin A; CHO, Chinese hamster ovary; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

tagenesis. Here we show genetic and biochemical evidence that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on EF-2, the target of bacterial ADP-ribosylating toxins.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—CHO-K1 cells were obtained from the American Type Culture Collection. CHO-K1 cells and mutants were maintained in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum, 2 mM glutamine, and antibiotics (penicillin and streptomycin).

Retroviral Gene Trap Insertional Mutagenesis—The retroviral gene trap vector, ROSAβgeo (20), was used to construct the random gene trap insertional mutants library of CHO cells. To infect the ROSAβgeo to CHO cells, pseudo-retrovirus of ROSAβgeo was produced using a pantropic retroviral expression system (Clontech) according to the manufacturer's instruction. The pROSAβgeo (20) and pVSV-G plasmid constructs were co-transfected to GP293 cells with Lipofectamine (Invitrogen). After 48-h incubation, virus-containing supernatant was harvested, passed through a 0.22-μm filter, and stored at −80 °C until use. For gene trap mutagenesis, CHO-K1 cells were seeded in multiple dishes at 1×10^6 cells per 100-mm dish. After overnight incubation, the medium was replaced with a virus-containing medium. After an additional 48 h, the cells from each dish were re-seeded at 1×10^6 cells per 100-mm dish, and gene trapped mutagenized cells were selected by G418 (200 μg/ml) for 10–14 days. Typically we could get $1–2 \times 10^3$ G418-resistant colonies per dish. Cells from $\sim 1 \times 10^6$ independent colonies were combined and stored as the random gene trap insertional mutants library of CHO cells.

Toxins—DT and fragment A of DT were purified by DEAE-cellulose column chromatography (14). ETA was purchased from List Biological Laboratories.

Selection and Cloning of Diphtheria Toxin-resistant Cells—Toxin-resistant CHO mutants were isolated from the gene trap insertional mutants library. Inocula of 10^6 gene trap mutagenized CHO cells were incubated for 6–10 h before the addition of 1 μg/ml of DT. After about 2 weeks of selection, colonies were isolated with cloning rings, and each isolated clone was cultured in DT-free medium.

Cytotoxicity Assay with MTT—Toxin-induced cytotoxicity was evaluated by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. Cells were seeded in 96-microwell plates at a density of 4×10^3/well in Dulbecco's modified Eagle's medium with 8% fetal calf serum. After 16-h incubation, the medium was replaced and cells were exposed to serial dilutions of DT or ETA (0–1000 ng/ml) for 48 h. Then 10 μl of 0.4% MTT reagent and 0.1 M sodium succinate were added to each well. After 90-min incubation, 150 μl of Me_2SO were added to dissolve the purple formazan precipitate. Formazan dye was measured spectrophotometrically (570–650 nm) using the MAXline Microplate Reader (Molecular Devices, Sunnyvale, CA) (11, 24).

Southern Blot Analysis—Genomic DNA was isolated from CHO cells using PUREGENE DNA isolation kits (Gentra), digested with EcoRI, separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane. A 0.8-kbp PCR fragment of the Neo^r gene, amplified with primers 5′-AACCATGGGATCGGCCATTGAACA-3′ and 5′-AGGATCCGCGAAGAACTCGTCAAGAAGGC-3′ from pROSAβgeo, was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) by random priming. The membrane was hybridized with this probe, washed, and then autoradiographed.

5′-Rapid Amplification of cDNA Ends (RACE), Cloning, and Sequence—5′-RACE analyses were conducted using the 5′-Full RACE core set (Takara Biomedicals) with total RNAs from DTR44 cells following the manufacturer's instructions. Total RNAs were prepared from mutant cells with Sepasol RNA I (Nacalai Tesque). Single strand cDNAs were prepared from the total RNAs (~1 μg of total RNA for each sample) with β-galactosidase-specific 5′-phosphorylated RT primer, 5′-ATGCGCTCAGGTCAAATTCA-3′ and avian myeloblastosis virus reverse transcriptase. After the degradation reaction of the hybridized RNAs by RNase H, cDNAs were circularized and/or concatemerized by using the 5′-Full RACE core set following the manufacturer's instructions. For PCR amplification of the trapped sequence, the following β-galactosidase-specific primers sets were used: 5′-GTTGATGAAAGCTGGCTACA-3′/5′-GTGCTGCAAGGCGATTAAGT-3′ (for the first PCR) and 5′-TGATGGCGTTAACTTGGCGT-3′/5′-TTCCCAGTCACGACGTTGTA-3′ (for nested PCR). Amplification products were subcloned to pGEM-T easy vector (Promega) and sequenced by using ABI PRISM™ 377 and 310 sequencing machines (Applied Biosystems). Homology of the trapped sequences was searched by the NCBI BLAST program.

PCR Analyses of Chimera RNA and Genome DNA—Total RNAs were reverse transcribed and amplified by using an RNA PCR kit (Takara Biomedicals). Genome DNAs were prepared from CHO cells using PUREGENE DNA isolation kits (Gentra). For PCR amplification of the cDNA and genome DNA, the following *Ovca1* and β-galactosidase-specific primers sets were used: 5′-CGTTCCTCCAGCGCTGCCTT-3′ (P1)/5′-GTGCTGCAAGGCGATTAAGT-3′ (P3) (for the first PCR) and 5′-TCCAGCGCTGCCTTTTTGGT-3′ (P2)/5′-TTCCCAGTCACGACGTTGTA-3′ (P4) (for nested PCR) (see Fig. 2, *B* and *C*).

cDNA Expression Experiments—The mouse *Ovca1* (*Ovca1* (m)), human *DPH2L2* (*DPH2L2* (h)), and mouse *dph2l2* (*dph2l2* (m)) cDNA expression vectors were constructed as described below. First, the cDNAs used in the expression experiments were RT-PCR amplified from the mouse kidney and human fibroblast mRNAs. The mRNAs were reverse transcribed with oligo dT primer and avian myeloblastosis virus reverse transcriptase. The mouse *Ovca1* cDNA fragments were amplified using 5′-primers for without tag *Ovca1* (5′-ATGCTAGCGT-**GATG**GCGGCGCTGGTTGTGT-3′ (the NheI site is underlined, and the start codon is in boldface)) or for with FLAG-tagged *Ovca1* (5′-ATGC-TAGC**ATG**GCGGCGCTGGTTGTGTC-3′ (the NheI site is underlined and the start codon is in boldface)) and a 3′-primer (5′-ATGGATCCC-CCTGCTGCTCTGGCCCTCTCA-3′ (the BamHI site is underlined)). The amplified cDNA fragments were then digested by NheI and BamHI and cloned into the NheI and BamHI sites of pIREShyg3 (Clontech) or pFLAG-IREShyg3. The pFLAG-IREShyg3 plasmid was constructed as follows. Oligonucleotides, 5′-AGCTTAAGTCCACC**ATG**GATTACAAG-GATGACGAC-3′ (the AflII site is underlined, and the start codon is in boldface) and 5′-TAGCTAGCGATCTTATCGTCGTCATCCTTGTAAT-C-3′ (the NheI site is underlined) were annealed, purified by PAGE, digested by AflII and NheI, and cloned into AflII and NheI sites of pIREShyg3. The mouse *dph2l2* cDNA fragment was amplified using a 5′-primer (5′-ATGCTAGCAAGCTGCGCCCA**ATG**GAGTCTA-3′ (the NheI site is underlined and the start codon is in boldface)) and a 3′-primer (5′-CTGATATCCAGACTTATCACTCTGGCTC-3′: the EcoRV site is underlined)). The amplified cDNA fragment was digested by NheI and EcoRV and cloned into the NheI and EcoRV sites of pIRES-Hyg3. The human *DPH2L2* cDNA fragment was amplified using a 5′-primer (5′-CATGGATCCCAAGCTGTGCCTC**ATG**GAGTC-3′ (the BamHI site is underlined and the start codon is in boldface) and a 3′-primer (5′-GATCTCGAGCACATGGTATCAGCCGCTTCC-3′ (the XhoI site is underlined)). The amplified cDNA fragment was digested by BamHI and XhoI and cloned into the BamHI and XhoI sites of pcDNA3.1/Hygro (+) (Invitrogen). These expression plasmid constructs were confirmed by restriction enzyme map and sequencing and were transfected into CHO cells using Lipofectamine reagent (Invitrogen). Stably transfected cells were selected by hygromycin B (400 μg/ml) for 10–14 days. Hygromycin-resistant colonies were isolated and analyzed.

Western Blot Analysis—CHO cells grown in 24-well plates were incubated with or without 1 μg/ml DT at 37 °C for 1 h, and the cells were washed and lysed by 100 μl/ml radioimmune precipitation assay buffer. Cell lysates were separated by native PAGE or SDS-PAGE and transferred to a nylon membrane (Hybond-P, Amersham Biosciences). Membranes were blocked in appropriate blocking buffers and incubated with goat anti EF-2 antibody (sc-13004, Santa Cruz Biotechnology) followed by peroxidase-conjugated anti-goat antibody. Reactive bands were detected by enhanced chemiluminescence (Amersham Biosciences).

In Vitro ADP-ribosylating Assay—CHO cell lysates preparation and ADP-ribosylation reaction of EF-2 were performed as described previously (15, 16). Cell lysates were incubated with [*adenylate*-^{14}C]NAD (Amersham Biosciences, 248 mCi/mmol, catalog no. CFA 497) in the absence or presence of DT fragment A. The amount of ADP-ribosylated EF-2 was assessed by counting the radioactivity incorporated to the acid insoluble fraction in a liquid scintillation counter (Aloka, LSC-3500). ADP ribosylation of EF-2 was confirmed by SDS-PAGE followed by autoradiography.

RESULTS

Isolation of the Diphtheria Toxin-resistant Mutants—The random gene trap insertional mutants library of CHO cells was made by infecting the ROSAβgeo (20) followed by growth in G418 as described under "Experimental Procedures." By combining the cells from $\sim 1 \times 10^6$ independent G418-resistant colonies, we constructed a library of mutants. The gene trap mutagenized cells were inoculated at 20×10^5 cells per

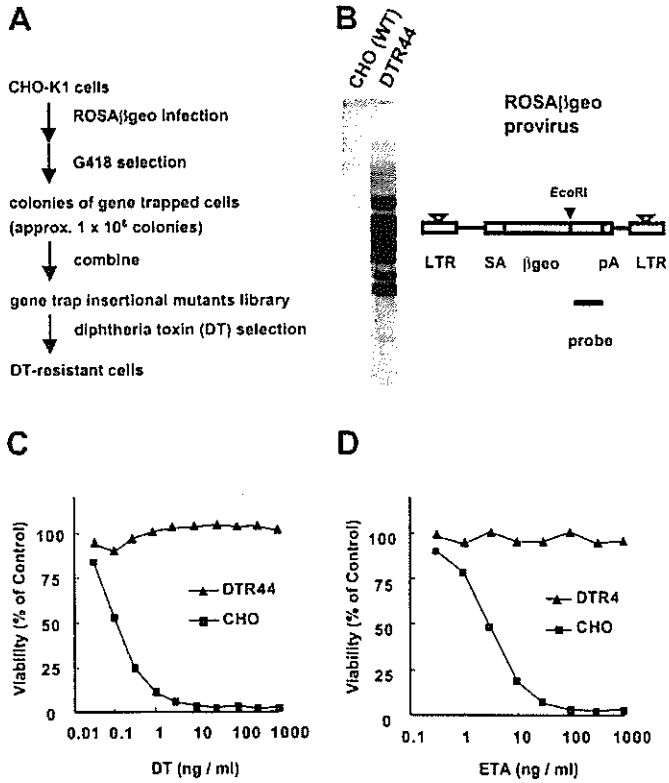

FIG. 1. **DTR44, obtained by retroviral gene trap insertional mutagenesis, is a multiple toxin-resistant mutant.** *A*, schematic representation of the construction of the random gene trap insertional mutants library of CHO cells and the isolation of the DT-resistant mutants. *B*, Southern blot analysis of the retroviral insertions in CHO cells. Genomic DNAs were digested with EcoRI, separated on 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled PCR-amplified Neo^r gene fragment. The structure of ROSAβgeo provirus (20) is also shown schematically. *LTR*, long terminal repeat; *SA*, splice acceptor; *βgeo*, β-galactosidase-Neo^r fusion gene; *pA*, polyadenylation signal. Cytotoxicity of DT (*C*) and ETA (*D*) to CHO cells. CHO cells were incubated with various concentrations of DT or ETA. After 48-h exposure to toxins, cell viability was determined by MTT assay as described under "Experimental Procedures."

100-mm culture dish and selected with DT. Colonies, ~10 per dish, were observed 10–14 days later. A total of 24 clones were picked randomly from the 1×10^6 mutant library cells (Fig. 1*A*). One of these mutants, DTR44, was completely resistant to DT and ETA (Fig. 1, *C* and *D*). Southern blot analysis with a Neo^r gene fragment as a probe showed multiple insertions (>8 copies) in the DTR44 genome (Fig. 1*B*).

Identification of the Disrupted Gene in DTR44 Cells—To identify the gene responsible for multiple toxin resistance in DTR44, we amplified the trapped sequences with a gene trap vector, ROSAβgeo, using 5′-RACE. A major PCR product was amplified from the DTR44 cDNA (data not shown) and 128-bp trapped sequences (DDBJ accession number AB194396) were clarified by sequencing. BLAST search revealed that 76 bp (number 53 to 128) of these 128-bp trapped sequences strongly matched those of the first exon of the mouse (25) and human *OVCA1* genes (Fig. 2*A*). The amino acids sequence of Chinese hamster OVCA1 protein in this region completely matched with that of mouse OVCA1, and 85% of these amino acids are identical to those of human OVCA1 protein (Fig. 2*A*).

The existence of the *Ovca1*-βgeo chimera RNA resulting from gene trap mutagenesis was confirmed by RT-PCR of DTR44 mRNA (Fig. 2*B*). Although multiple insertions of the gene trap vector into DTR44 genome were ascertained by Southern blot (Fig. 1*B*), PCR sequence analysis of the DTR44 genomic DNA with the primers correspond to *Ovca1*, and βgeo sequences clarified that one of the gene trap retrovirus was integrated within the first intron, ~1kb downstream of exon 1, of the *Ovca1* gene (Fig. 2*C*).

Disruption of the Ovca1 Gene Renders a Toxin-resistant Phenotype—To define the role of OVCA1 in DTR44, *Ovca1* cDNA expression plasmids were constructed and transfected into DTR44 cells; stable transformants were then established by selecting with hygromycin B (400 μg/ml). Stable transfectant colonies were picked and evaluated for sensitivity to the toxins. The majority of clones arising from DTR44 cells transfected with *Ovca1* cDNA regained sensitivity to DT and ETA (Fig. 3*A*). In contrast, in cells transfected with the empty vector or DPH2L2, proteins that have higher similarity to DPH2 than OVCA1 (26), expression vector remained toxin-resistant (Fig. 3*A*).

Cytotoxicities of DT or ETA to DTR44 cells and the clones of DTR44 transfected with OVCA1 expression constructs were further analyzed by MTT assay. Clones R1 and R2, DTR44 stable transformants of mouse *Ovca1* cDNA expression vector, regained their parental sensitivity and were fully sensitive to DT and ETA (Fig. 3, *C* and *D*).

The presence of *Ovca1*-βgeo chimera RNA in these transformants (Fig. 3*B*) clearly showed that the recovery of toxin sensitivity was not caused by the deletion of the gene trap vector integrated in the *Ovca1* gene. Taken together, we concluded that OVCA1 was required for the process involved in sensitivity to DT and ETA.

OVCA1 Is Required for Diphthamide Biosynthesis—DTR44 cells showed the characteristics of the DT^{R}II phenotype, that is, multiple toxin resistances and a resistant to high concentrations of toxins. DT^{R}II mutants have shown that DT sensitivity was affected at the level of EF-2 (8, 12, 13, 15–18).

To determine whether DTR44 cells are altered in their susceptibility of EF-2 to ADP-ribosylation, CHO cell extracts were assayed for transfer of radiolabeling from NAD^+ to EF-2. Lysates were incubated with [*adenylate*-^{14}C]NAD in the absence or presence of DT fragment A. The amount of ADP-ribosylated EF-2 was assessed by counting the radioactivity incorporated to the acid insoluble fraction with a liquid scintillation counter (Fig. 4*A*). ADP ribosylation of EF-2 was confirmed by SDS-PAGE followed by autoradiography. (Fig. 4*B*).

ADP-ribosylation assay clearly showed that ADP-ribosylated acceptor activity in DTR44 cell lysate was dramatically reduced compared with that of wild type parental CHO cells (Fig. 4, *A* and *B*). Furthermore, the non-ribosylatable EF-2 from DTR44 cells restored the ADP-ribosyl acceptor activity when transfected with OVCA1 expression vector (Fig. 4, *A* and *B*). From these observations, we concluded that DTR44 cells are defective in diphthamide formation on EF-2 and that OVCA1 is required for the biosynthesis of diphthamide.

Identification of Intermediate in Diphthamide Synthesis—ADP-ribosylated, the non-ADP-ribosylated form, and biosynthetic intermediate of diphthamide on EF-2 have been shown to be easily distinguished by native PAGE followed by Western blotting using an anti-EF-2 antibody (11). Using this detection system, we examined the diphthamide biosynthetic intermediate in DTR44 cells. ADP-ribosylated EF-2 has two added negative charges compared with EF-2, and this increased negative charge could be detected as faster migration on native PAGE. The EF-2 of DTR44 migrates between the ADP-ribosylated and non-ADP-ribosylated forms (Fig. 4*C*). This observation further confirmed that OVCA1 is required for the biosynthesis of diphthamide on EF-2, the target site for the ADP-ribosylating bacterial protein toxins.

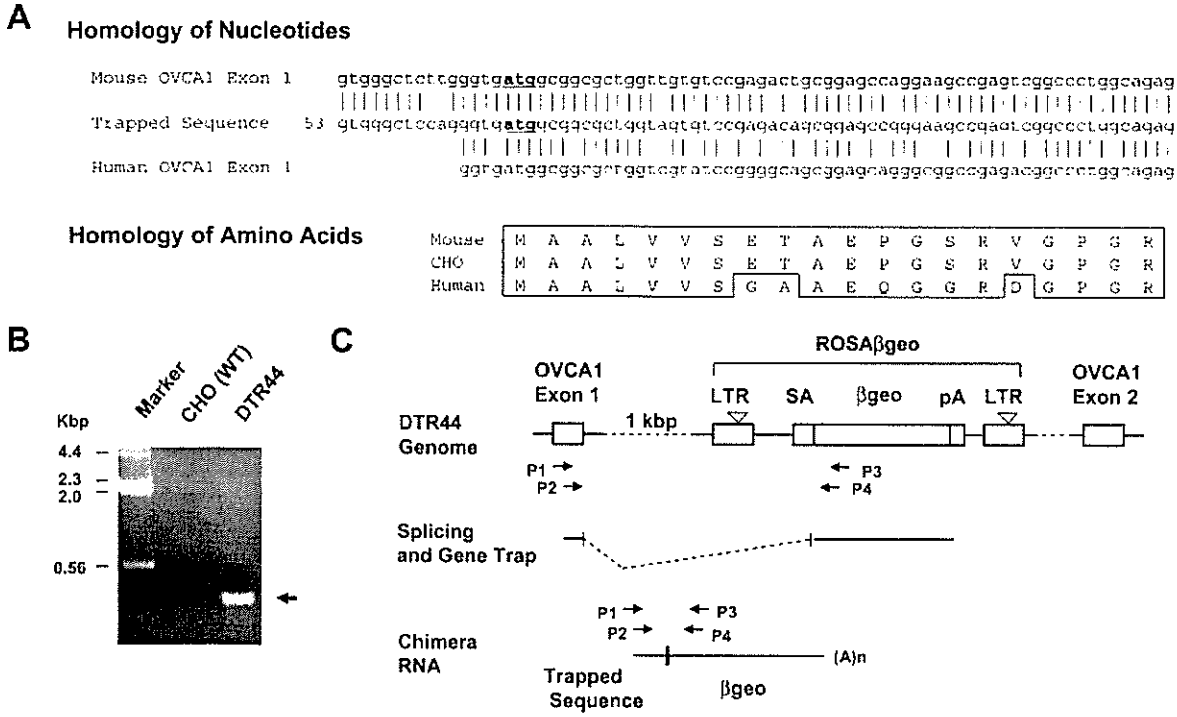

FIG. 2. **The tumor suppressor *Ovca1* gene was disrupted by gene trap insertional mutagenesis in DTR44 mutant.** *A*, the nucleotides sequences (DDBJ accession number AB194396) trapped by ROSAβgeo in DTR44 cells were elucidated by 5'-RACE and sequence analyses of DTR44 cDNA. BLAST homology search revealed that the first exon of *Ovca1* gene was trapped in DTR44 mutant cells. Homologies of the nucleotides and the deduced amino acids of mouse, Chinese hamster (CHO cells), and human OVCA1 in this region are shown. *B*, *Ovca1*-βgeo chimera RNA produced by gene trap event was confirmed in DTR44 mutant cells by RT-PCR as described under "Experimental Procedures." The *arrow* indicates the 211-bp RT-PCR (nested PCR) product amplified using primers P2 and P4 from *Ovca1*-βgeo chimera-RNA in DTR44 cells. *C*, schematic representation of gene trap event in the *Ovca1* gene of DTR44 cells. Gene trap retrovirus was integrated in the first intron of *Ovca1* gene. PCR-sequence analyses of the DTR44 genomic DNA with the primers corresponding to *Ovca1* and βgeo sequences (P1/P3 and P2/P4) clarified that one of the gene trap retrovirus was integrated in the first intron, ~1kb downstream of exon 1, of the *Ovca1* gene (data not shown).

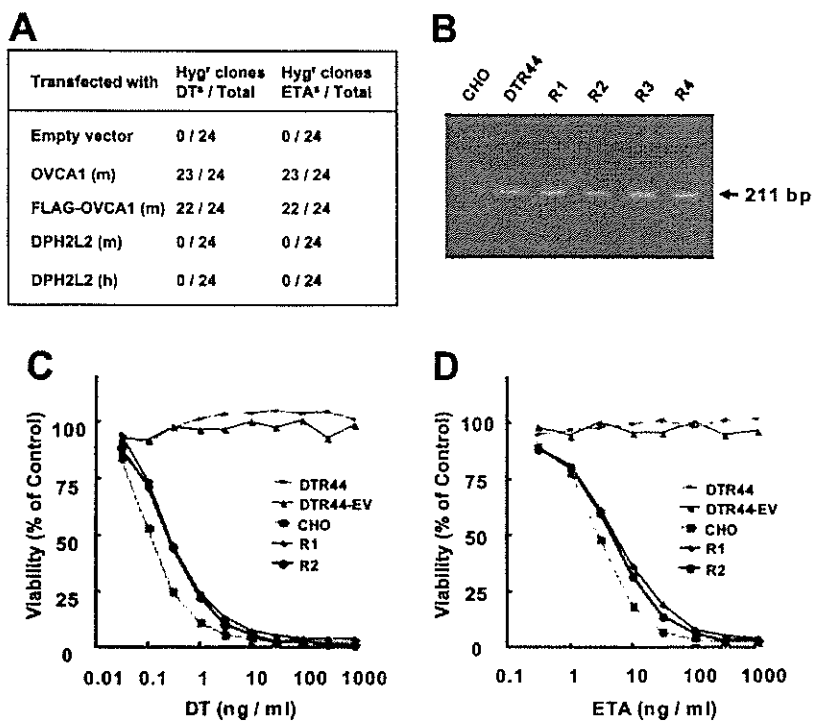

FIG. 3. **Expression of OVCA1 restores the sensitivity of DTR44 mutant cells to bacterial ADP-ribosylating toxins.** *A*, effect of OVCA1 or DPH2L2 expression on sensitivity to toxins. The expression plasmids of the indicated genes, mouse *Ovca1* (*OVCA1* (m)), FLAG-tagged mouse *Ovca1* (FLAG-*OVCA1* (m)), mouse *dph2l2* (*DPH2L2* (m)), and human *DPH2L2* (*DPH2L2* (h)) were transfected to DTR44 mutant cells, and stably transformed cells were established through growth for 10–14 days in hygromycin B (400 μg/ml). The cell colonies were isolated and subcultured to 48-well plates and then treated with DT (1 μg/ml) or ETA (1 μg/ml) for 96 h. The numbers of toxin-sensitive mutants (DT^s, DT-sensitive; ETA^s, ETA-sensitive) *versus* those that tested the sensitivities to toxins were determined by microscopic observation. *B*, RT-PCR analysis of the *Ovca1*-βgeo chimera RNA. *Ovca1*-βgeo chimera RNA was examined in CHO cells as in Fig. 2*B*. R1 to R4 are DTR44 cells transfected with mouse OVCA1 expression constructs and confirmed the recovery of sensitivity to toxins. R1 and R2 clones were used for further analyses. Cytotoxicities of DT (*C*) or ETA (*D*) to CHO cells were determined by MTT assay as in Fig. 1. DTR44-EV is a stably transformed DTR44 cells transfected with an empty vector, pIREShyg3.

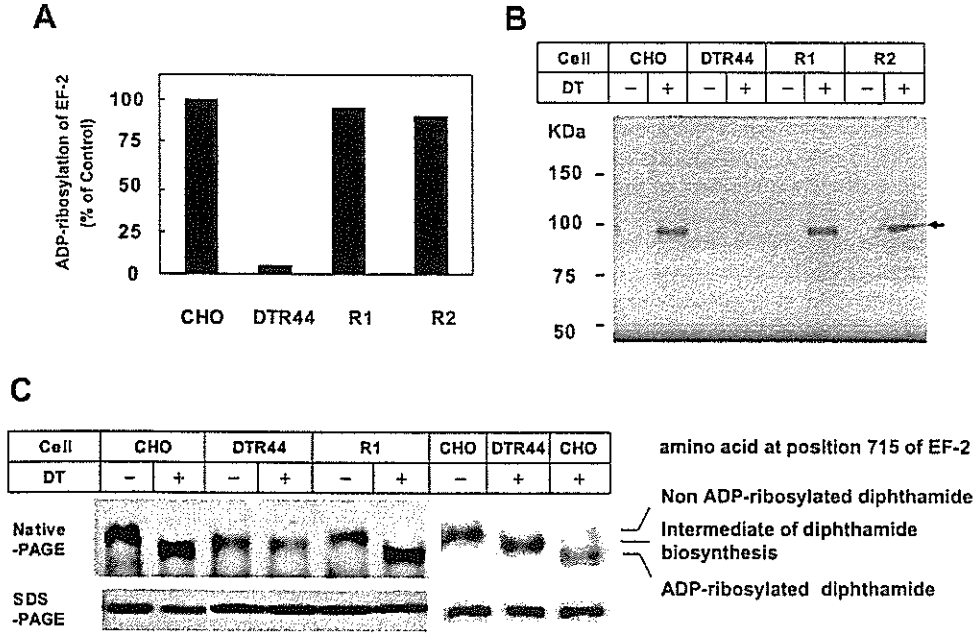

FIG. 4. **DTR44 mutant cells are defective in diphthamide formation of EF-2.** CHO cell lysates were incubated with [*adenylate*-^{14}C]NAD in the absence or presence of DT. *A*, *in vitro* ADP-ribosylating assay. EF-2 of DTR44 cells is resistant to ADP-ribosylation by DT. The amount of ADP ribosylated EF-2 *in vitro* was assessed by counting the radioactivity incorporated to the acid insoluble fraction as described previously (15, 16). R1 and R2 are DTR44 clones transfected with mouse OVCA1 expression vector. *B*, ADP-ribosylation of EF-2 *in vitro* was confirmed by SDS-PAGE followed by autoradiography. One major band (~95 kDa) corresponding to ADP-ribosylated EF-2 was observed. EF-2 of CHO, R1, and R2 cells were the ADP-ribosylatable form. The non-ribosylatable EF-2 in DTR44 cells could be converted to the ADP-ribosylatable form by transfection of cells with OVCA1 expression vector. *C*, Western blots analyses of the EF-2. After 1 h incubation with or without 1 μg/ml of DT at 37 °C CHO cells were lysed by radioimmune precipitation assay buffer, and cell lysates were separated by native PAGE (*top*) or SDS-PAGE (*bottom*) followed by Western blots analyses using an anti-EF-2 antibody as described under "Experimental Procedures."

DISCUSSION

To elucidate the molecular mechanisms underlying DT sensitivity and/or resistance, including diphthamide biosynthesis, we screened mutants resistant to DT from a library of random gene trap insertional mutants of CHO cells. CHO cells are functionally hemizygous at a number of loci (22, 23). It was expected that a single integration event of gene trap retrovirus might result in the loss of gene function in CHO cells, and we would be able to get mutants with specific phenotype by proper selection from this library of mutant cells.

DTR44 mutant cells isolated from the gene trap insertional mutants library showed a phenotype with multiple toxin resistance to DT and ETA. It was revealed that the *Ovca1* gene was disrupted by gene trap mutagenesis and that the expression of OVCA1 fully recovered the sensitivity to toxins and EF-2 diphthamide formation. These genetic and biochemical data clearly show that the tumor suppressor OVCA1 is a component of the biosynthetic pathway of diphthamide on EF-2.

So far the amino acid sequences of OVCA1 did not reveal any functional protein domains suggesting its biochemical function except for low level sequence similarity (~20%) (1, 2) between OVCA1 and DPH2, a yeast protein necessary for diphthamide formation (10). The forward genetic approach using gene trap mutagenesis in this study clearly demonstrated the biochemical function of OVCA1 in the formation of diphthamide in mammalian cells as described above.

The retroviral gene trap mutagenesis approach described in this study is relatively simple and straightforward. Even though multiple copies of gene trap vectors are integrated into DTR44 mutant genome (Fig. 1*B*), we could easily identify the obligate gene for DT-resistant phenotype in DTR44 cells by analyzing the trapped sequences in the chimera RNA produced by gene trap mutagenesis. Retroviruses can be used as insertional mutagens to isolate specific genes in mammalian cells. However, in practice, conventional retroviruses are inefficient mutagens (27). In ROSAβgeo, the retroviral gene trap vector used in this study, βgeo is flanked by an upstream 3′-splice consensus sequence (splice acceptor) and a downstream polyadenylation site to ensure its activation from integrations into introns ("intron trap"), and the gene trap events by SAβgeo are estimated to ~4.5 to 11.6% of integration events (20). So even if multiple integration events occurred, it was estimated that a large portion of retroviral vector integrations were not involved in the specific phenotypes.

After the isolation of the *Ovca1* gene (1, 2), the biological functions of OVCA1 were clarified by cell biological analyses and the study of gene knock-out mice. It was speculated that the loss or haploinsufficiency of OVCA1 might be an important event in ovarian tumorigenesis from the observation that expression of OVCA1 protein in ovarian tumor tissues or cell lines was reduced (3). It was also seen that exogenous expression of OVCA1 in ovarian cancer cells causes suppression of cell growth with an increased number of cells in G_1 phase of the cell cycle, suggesting that OVCA1 may play a role in the control of cell cycle/cell growth (3). Furthermore, study using knock-out mice demonstrated that OVCA1 regulates cell proliferation, embryonic development, and tumorigenesis (4).

The fact that the tumor suppressor OVCA1 is involved in diphthamide biosynthesis on EF-2 suggests the possibility that aberrations in translational regulation may be one of the molecular mechanisms underlying the tumorigenesis caused by the defect of OVCA1. So far it has been elucidated that components of the protein synthesis apparatus seem to be involved in the control of cell proliferation, and aberrations in protein synthesis are commonly encountered in established cancers (28). Furthermore, it has been demonstrated that removal of regulation of the expression of components of the translational machinery, such as elongation factor-1 α, a GTP-binding protein that catalyzes the binding of aminoacyl-transfer RNAs to

the ribosome, predispose cells to become more susceptible to malignant transformation (29).

It has also been demonstrated that the activity of EF-2 kinase was markedly increased in several forms of malignancies and that inhibition of EF-2 kinase inhibited the growth of a variety of cancer cell lines (28, 30–32). Phosphorylation of EF-2 by EF-2 kinase results in a drastic inhibition of protein synthesis, and dephosphorylation of EF-2 by phosphatase restores its activity. The phosphorylation of EF-2 directly affects the elongation stage of translation, and this represents a novel mechanism of translational control (33). It is possible to speculate that the defect of OVCA1 also disturbs the translational regulation through the abnormal diphthamide formation on EF-2 and results in the cause of tumorigenesis.

The fact that OVCA1 is a component of diphthamide biosynthetic pathway may also provide an important clue for a better understanding of the biological function of diphthamide. The biosynthesis of diphthamide represents one of the most complex post-translational modifications of an amino acid known to date and is widely well conserved (5, 6), suggesting that it has real importance for biological function. However, the function and role of diphthamide in cellular physiology still remains obscure.

Thus far, the existence of endogenously ADP-ribosylated EF-2 and cellular ADP-ribosyltransferase activity has been found in a variety of animals and tissues. The enzyme transfers ADP-ribose from NAD to elongation factor 2, inactivating the factor like bacterial toxins (34–36). However, the nature of the cellular ADP-ribosyltransferase and its physiological significance remain unknown. To clarify that the effect on cell proliferation, embryonic development, and tumorigenesis observed in the *Ovca1* knock-out mice (4) truly result from the defect of diphthamide, the generation of mice lacking other genes in the diphthamide biosynthesis may be helpful.

The finding that OVCA1 is a component of the diphthamide synthetic pathway will shed light for the further understanding of the function of OVCA1, molecular mechanisms underlying the tumorigenesis in the defect of OVCA1, and the physiological role of diphthamide. Furthermore, a functional genetic approach utilizing the random gene trap mutants library of CHO cells described above should become a useful strategy to identify the genes responsible for specific phenotypes.

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