Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane

Tadashige Nozaki,[1] Hisako Fujihara,[1] Masatoshi Watanabe,[2] Masahiro Tsutsumi,[3] Kentaro Nakamoto,[1] Osamu Kusuoka,[3] Nobuo Kamada,[4] Hiroshi Suzuki,[4,5] Hitoshi Nakagama,[1] Takashi Sugimura[1] and Mitsuko Masutani[1,6]

[1]Biochemistry Division, National Cancer Center Research Institute, Chuo-ku, Tokyo 104-0045, [2]Department of Pathology, Mie University School of Medicine, 2-174 Edobashi, Tsu City, Mie 514-8507, [3]Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, [4]Chugai Pharmaceutical Co., Ltd., Gotemba, 1-135 Komakado, Gotemba, Shizuoka 412-0038

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Poly(ADP-ribose) polymerase-1 (Parp-1) is activated by DNA strand breaks and functions in the maintenance of genomic integrity and cell death control. On the other hand, Parp-1 is also involved in transcriptional regulation of various genes, and the relationship between Parp-1 deficiency and susceptibility to tumorigenesis has not been fully elucidated. In the present study, *Parp-1*$^{-/-}$ mice, harboring exon 1 disruption in *Parp-1*, and *Parp-1*$^{+/+}$ animals were administered azoxymethane (AOM) at a dose of 10 mg/kg body weight once a week for 6 weeks. At 30 weeks after the first carcinogen treatment, mice were sacrificed. The incidence of animals bearing either adenomas or adenocarcinomas in the colon and the average number of colon tumors per mouse were significantly higher in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ animals. β-Catenin accumulation was observed in 43/44 of *Parp-1*$^{-/-}$ tumors and 19/21 of the *Parp-1*$^{+/+}$ tumors and was not statistically different between the genotypes. This suggests that most tumors developed through a pathway involving the alteration of Wnt-β-catenin signaling in both *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice. In the liver, where AOM is primarily activated, the incidence of animals bearing nodules and the average number of nodules per section were significantly increased in *Parp-1*$^{-/-}$ mice compared with *Parp-1*$^{+/+}$ mice. Therefore, the results indicate that susceptibility to AOM-induced tumorigenesis in the colon and also in the liver is enhanced in *Parp-1*$^{-/-}$ mice, and Parp-1 could have a substantial role in colon and liver tumorigenesis. (Cancer Sci 2003; 94: 497–500)

The polyADP-ribosylation reaction is catalyzed by poly(ADP-ribose) polymerase (Parp) family proteins using NAD as a substrate. Parp-1 is a 113-kDa nuclear enzyme which polyADP-ribosylates various nuclear proteins, including Parp-1 itself and histones, after activation by binding to DNA single and double strand breaks. *Parp-1*$^{-/-}$ cells show delayed DNA rejoining after treatment with alkylating agents, and a role of Parp-1 in base excision repair and double strand break (DSB) repair has been suggested.[1] *Parp-1*$^{-/-}$ cells also display enhanced genomic instability, including chromosome aberration and increased levels of micronuclei formation both in the absence of DNA damage and after DNA damage introduction.[2,3] In addition, Parp-1 also has a leading role in cell death after DNA damage and Parp-1 overactivation. NAD depletion after DNA damage was abrogated in *Parp-1*$^{-/-}$ cells and as a consequence, *Parp-1*$^{-/-}$ cells became resistant to cell death induced by severe DNA damage.[4–8] This evidence suggests that loss of Parp-1 function could have a substantial contribution to tumorigenesis, through the introduction of genetic alterations.

In fact, we previously demonstrated that *Parp-1*$^{-/-}$ mice, harboring exon 1 disruption in *Parp-1*, showed increased incidences of liver and lung tumors after *N*-nitrosobis(2-hydroxypropyl)amine administration.[9] Pronounced susceptibility to tumorigenesis was also reported with *Parp-1*$^{-/-}$ SCID by Morrison *et al.*[10] and with *Parp-1*$^{-/-}$ *p53*$^{-/-}$ mice[11] and *Parp-1*$^{-/-}$ *Ku80*$^{-/-}$ mice,[12] harboring exon 2 disruption in *Parp-1*, by Tong *et al.*[13] It is also known that pharmacological intakes of niacin augment NAD level and increase the latency of the ethylnitrosourea-induced liver carcinogenesis.[14] Since *Parp-1* is also involved in the transcriptional regulation of various genes,[15–17] the impact of Parp-1 deficiency may differ among various types of tumors and among various organs. Therefore, the effect of Parp-1 deficiency on tumorigenesis needs to be investigated in various models of tumorigenesis.

In the present study, *Parp-1*$^{-/-}$ mice, harboring exon 1 disruption of *Parp-1*, were treated with azoxymethane (AOM), a potent colon carcinogen. We observed that the *Parp-1*$^{-/-}$ mice showed an enhanced incidence of tumor development in the colon and also in the liver. Colon tumors induced by AOM in rodents are frequently associated with alteration of the Wnt-β-catenin signaling cascade, and β-catenin accumulation is observed.[18] Therefore, to examine whether the development of tumors in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice involves alteration of Wnt-β-catenin signaling, immunostaining of β-catenin was carried out.

Materials and Methods

Parp-1$^{-/-}$ mice generated by disrupting the *Parp-1* exon 1 through the insertion of a neomycin resistance gene cassette[5] were used in this study. Mice were housed in plastic cages in an air-conditioned room with a 12 h light-dark cycle. Water and basal diet (CE-2, CLEA JAPAN, Tokyo) were available *ad libitum*. *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ female mice (7–8 weeks old) of a mixed genetic background of ICR and 129Sv were produced by line-breeding. The mean body weights of *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice at the start of the experiment were 21.7±1.7 g and 21.4±2.9 g, respectively. *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice received i.p. injections of either AOM (Sigma, St. Louis, MO) in sterile saline at a dose of 10 mg/kg body weight or saline alone once a week for 6 weeks. Mice that became moribund before the termination of experiment were euthanized and autopsied, and dead animals were necropsied. Thirty weeks after the first carcinogen treatment, the remaining mice were euthanized and all organs were examined macroscopically for the presence of tumors and fixed in neutralized 10% formalin solution. The colons were opened along the longitudinal axis and the number and maximum diameter of colon tumors detectable with naked eyes were measured. Locations of colon tumors were determined after dividing the colons equally into proximal, middle and distal parts. Histological analysis of colon tumors was performed after the routine process of embedding the tissue in paraffin and staining sections with hematoxylin and eosin. In addition to ordinary polypoid tumors, flat adenomas were de-

[6]To whom correspondence should be addressed.
E-mail: mmasutan@gan2.res.ncc.go.jp
[5]Present address: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2-13 Nishi, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.

fined as previously described.[19] Liver sections were prepared in 5 mm thickness from each lobe at maximum diameter, and histological analysis of the liver tumors was carried out as described by Frith and Ward.[20, 21] The number of liver nodules on each section was analyzed according to the criteria of Campbell *et al*.[22] The maximum diameters and areas of nodules were analyzed with the aid of an image analyzer, IPAP-WIN software, version 1.0.0.1 (Sumika Technoservice Corp., Osaka).

For β-catenin immunostaining, tissue sections prepared at 3.5 μm thickness were mounted on slides coated with aminosilane (Matsunami, Osaka), deparaffinized with xylene, and rehydrated with graded alcohol. Autoclave treatment in citrate buffer was used for antigen retrieval. Immunostaining of the sections with a monoclonal antibody against mouse β-catenin (Transduction Lab., Lexington, KY) diluted at 150-fold in PBS containing 2% goat serum and 0.1% BSA was performed using the Ventana NX automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ). Sections processed without primary antibodies were used as negative controls.

The significance of differences was analyzed by the χ^2 test for the incidence, size distribution and locations of tumors and by Mann-Whitney *U* test for the number of tumors per mouse using SPSS for Macintosh (SPSS, Inc., Tokyo). To analyze differences in the number of liver nodules, Student's *t* test was used.

Results

Tumorigenesis in the colon. Administration of AOM did not affect body weight increase either in *Parp-1*$^{+/+}$ or *Parp-1*$^{-/-}$ mice. Data for colon tumor development are summarized in Table 1. *Parp-1*$^{-/-}$ mice treated with AOM showed a significantly higher incidence of the colon tumors compared with that of *Parp-1*$^{+/+}$ mice ($P<0.05$). The number of tumors per mouse was also higher in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ animals ($P<0.05$). No colon tumor was found in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice that received saline alone. The tumors were mostly well-differentiated or moderately differentiated adenocarcinomas and a few adenomas were observed. The incidences of adenocarcinomas were 21/21 and 41/44 in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice, and those of adenomas were 0/21 and 3/44 in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice, respectively. The tumors were mainly located in the distal part of the colon both in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice. The average of the maximum diameter of the colon tumors was larger in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ mice ($P<0.05$). Tumors were mostly of the polypoid type (Fig. 1A) and flat tumors were only infrequently observed, namely 2/21 (9.5%) of *Parp-1*$^{+/+}$ tumors and 4/44 (9.1%) of *Parp-1*$^{-/-}$ tumors. The majority of the tumors, 19/21 of the *Parp-1*$^{+/+}$ tumors and 43/44 of the *Parp-1*$^{-/-}$ tumors, exhibited dense β-catenin accumulation in the nuclei or cytoplasms in the tumors, as shown in Fig. 1. Nineteen of 19 polypoid tumors and 0/2 flat tumors in *Parp-1*$^{+/+}$ mice and 39/40 polypoid tumors and 4/4 flat tumors in *Parp-1*$^{-/-}$ mice showed β-catenin staining.

It was proposed that lymphoid tissues in the distal colon play a promotional role following carcinogen treatment.[23] No difference in the distribution of the gut-associated lymphoid tissues in the colon was observed between *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice before and after AOM treatment (data not shown).

Tumorigenesis in the liver. AOM is primarily metabolized and activated in the liver and *Parp-1*$^{-/-}$ mice displayed an increased incidence of liver nodules compared with *Parp-1*$^{+/+}$ mice after

Table 1. Colon tumor in mice given AOM

Mice	AOM dose (mg/kg BW)	No. of mice bearing tumors (%)	No. of tumors per mouse[2)]	No. of tumors				
				Maximum diameter		Location		
				<1.5 mm	≥1.5 mm	Distal	Middle	Proximal
Parp-1$^{+/+}$	0	0/23 (0)	0	0	0	0	0	0
	10	8/19 (42)	1.1±2.0	9	12	20	1	0
Parp-1$^{-/-}$	0	0/19 (0)	0	0	0	0	0	0
	10	16/21 (76)[1)]	2.1±1.8[1)]	8	36[1)]	40	4	0

1) Significantly different between *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice at $P<0.05$.
2) Mean±SE.

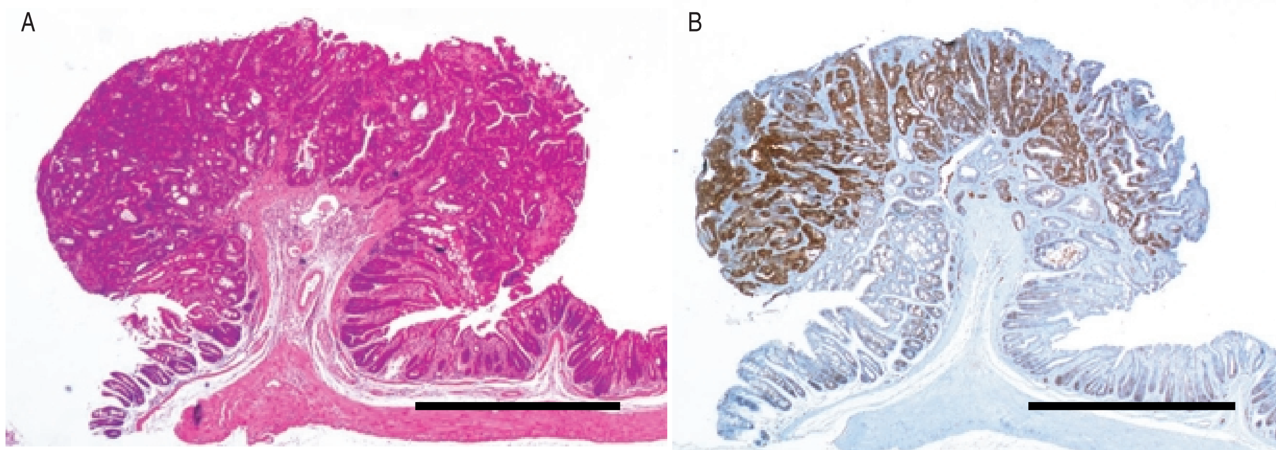

Fig. 1. Typical β-catenin-positive colon adenocarcinoma in *Parp-1*$^{-/-}$ mice. A. Hematoxylin-eosin staining of the tumor. B. Immunostaining with anti-β-catenin antibody. Bars indicate 1 mm.

Table 2. Liver nodule formation in mice given AOM

Mice	AOM dose (mg/kg BW)	Liver nodules		
		No. of mice bearing nodules (%)	No. of nodules[3] per section	Mean area (mm^2)
Parp-1$^{+/+}$	0	0/22 (0)	0	0
	10	2/19 (11)	0.055±0.35	1.19[3]
Parp-1$^{-/-}$	0	0/19 (0)	0	0
	10	9/22 (41)[1]	2.6±1.3[2]	0.29±0.19[4]

1) Significantly different between *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice at $P<0.05$.
2) Significantly different between *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice at $P<0.01$.
3) The size of two nodules was 0.39 and 2.0 mm^2, respectively.
4) Mean±SE.

AOM administration. The nodules are composed of cells, which resemble normal hepatocytes but are compressed compared to the surrounding liver tissue, as previously described.[19, 20] Nuclear atypia, mitoses, and apoptosis were not frequently observed. The trabecular and columnar patterns of plates of hepatic cells were maintained and there was no obvious disruption of the liver architecture, although the demarcation of nodules to adjacent normal parenchyma was detectable. Table 2 summarizes the liver nodule development in *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice after AOM treatment. Compared with *Parp-1*$^{+/+}$ mice, *Parp-1*$^{-/-}$ mice showed a significantly higher incidence of liver nodule formation ($P<0.05$) and the number of nodules per section ($P<0.01$). There were only two nodules in *Parp-1*$^{+/+}$ mice, so statistical analysis of the difference in size of the nodules between *Parp-1*$^{+/+}$ and *Parp-1*$^{-/-}$ mice was not carried out. β-Catenin accumulation in the liver nodules was not observed in either the *Parp-1*$^{+/+}$ or *Parp-1*$^{-/-}$ animals (data not shown).

Discussion

The present study demonstrates the increased susceptibility of *Parp-1*$^{-/-}$ mice to AOM-induced tumorigenicity compared with *Parp-1*$^{+/+}$ mice. The incidence of tumors both in the colon and liver after AOM treatment was significantly higher in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ mice. In contrast, the development of spontaneous tumors was not observed either in *Parp-1*$^{-/-}$ mice 9 months after birth, or in their *Parp-1*$^{+/+}$ counterparts, as previously described.[3, 5, 24] Since the histopathological features of the *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ colon tumors induced by AOM were similar to each other and the tumors of both genotypes showed accumulation of β-catenin, the results suggest that the majority of tumors of both genotypes developed through a common molecular pathway, namely alteration of the Wnt-β-catenin signaling. On the other hand, the average size of the colon tumors was larger in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ mice. Since *Parp-1*$^{-/-}$ cells show altered gene expressions,[25] the deficiency of Parp-1 could have influenced the transcription of genes involved in tumor growth control. In addition, *Parp-1*$^{-/-}$ embryonic fibroblasts become resistant to apoptosis inducing factor-dependent apoptosis induced by DNA damage.[26] The lower sensitivity to cell death after DNA damage under conditions of Parp-1 deficiency may potentially enhance tumor growth, as well as allowing the accumulation of genetic alterations in the cells.

In the liver, the incidence of liver nodules, precancerous lesions,[19] was also higher in *Parp-1*$^{-/-}$ mice than in *Parp-1*$^{+/+}$ mice given AOM. Therefore, under Parp-1 deficiency, the livers are also exposed to an increased risk of AOM-induced tumorigesis. AOM injection into rats induces tumors in the kidney, as well as in colon and liver.[27, 28] We thus examined tumor development in the kidney in *Parp-1*$^{+/+}$ or *Parp-1*$^{-/-}$ mice treated with AOM, but found no tumor in these mice.

AOM is metabolized mainly in the liver to methylazoxymethanol (MAM) and MAM spontaneously decomposes to methyldiazonium, OH^- and formaldehyde.[29] Methyldiazonium is further decomposed to a methyl cation, which induces DNA damage, including formation of adducts such as O^6-methylguanine and 3-methyladenine. Methyl adducts on DNA also evoke DNA strand breaks through abortive mismatch-repair process.[30] In addition, formaldehyde induces genetic alterations, such as sister chromatid exchanges and micronuclei formation.[31, 32] MAM is also conjugated to glucuronate by detoxification enzymes in the liver. The enterohepatic circulation delivers the glucuronate conjugate of MAM to the colon, where microbial glucuronidases release MAM. Multiple roles of Parp-1 in the repair of such DNA damage have been suggested. Parp-1 interacts with DNA-dependent protein kinase and Ku80[33, 34] and is involved in DSB repair. Parp-1 also protects single strand breaks and DSBs from accidental recombination through its anti-recombinogenic property and binding ability to DNA strand breaks. The association of Parp-1, XRCC-1 and DNA polymerase β has been reported, and Parp-1 may function as a key molecule in base-excision repair.[1, 35] Delay in DNA strand-break repair and/or base-excision repair in the Parp-1-deficient state possibly increases genetic alterations during tumorigenesis.

The susceptibility of *Parp-1*$^{-/-}$ mice to tumorigenesis has been reported using only a limited number of experimental systems. *Parp-1*$^{-/-}$SCID mice showed a higher incidence of thymic lymphoma compared with *Parp-1*$^{+/+}$SCID mice.[10] Similarly, *Parp-1*$^{-/-}$*p53*$^{-/-}$ mice, harboring exon 2 disruption in *Parp-1*, displayed increased incidences of various tumors compared with *Parp-1*$^{+/+}$*p53*$^{-/-}$ mice, including brain tumors, which were not observed in their *Parp-1*$^{+/+}$ counterparts.[11, 13] On the other hand, *Parp-1*$^{-/-}$*p53*$^{-/-}$ mice, harboring exon 4 disruption in *Parp-1*, showed delayed onset of tumorigenesis compared with *Parp-1*$^{+/+}$*p53*$^{-/-}$ animals.[36] *Parp-1*$^{-/-}$ mice, harboring exon 1 disruption in *Parp-1*, showed increased incidences of hemangiomas and hemangiosarcomas in the livers and those of adenomas in the lungs after *N*-nitrosobis(2-hydroxypropyl)amine administration.[9] Differences in the disrupted exon in the *Parp-1* gene and/or the presence of a modifier gene may cause this difference in the susceptibility to tumorigenesis.

The present model of AOM-induced tumorigenesis in *Parp-1*$^{-/-}$ mice should be useful to elucidate mechanisms of the colon and liver tumor development.

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