NOTE *Laboratory Animal Science*

Background Lesions during a 24-Month Observation Period in Connexin 32-Deficient Mice

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ABSTRACT. Connexin 32 (Cx32) is a major gap junction protein in the liver. Neoplastic and non-neoplastic lesions were examined in Cx32-deficient (Cx32KO) mice maintained for 24-month, and compared with those in wild-type mice as a corresponding control. In neoplastic lesions, hepatocellular carcinoma increased significantly only in male Cx32KO mice, suggesting that Cx32 deficiency may be related to their pathogenesis. For females, the incidence of pituitary adenoma in the pars distalis of Cx32KO mice was lower than that of wild-type mice. No non-neoplastic lesions related to Cx32-deficiency were observed in the Cx32KO mice. In conclusion, these results demonstrate that the incidence of hepatocellular carcinoma increases only in male Cx32KO mice, presumably due to enhanced tumor promotion and progression signals associated with Cx32 deficiency.

KEY WORDS: connexin 32, intercellular communication, knock-out mice, spontaneous mass.

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Connexin 32 (Cx32) is one of the major gap junction proteins mainly existing in the liver as well as minimum expressions in other organs, and is thought to play an important role in maintaining tissue homeostasis through gap junction intercellular communication (GJIC). Cx32 is also recognized to be involved in cell growth, proliferation and differentiation [10, 23]. Cx32 disruption was reported to induce dysfunction in several organs, namely, lower glucose mobilization in the liver, increased secretion of amylase from the exocrine pancreas and demyelinization in the peripheral nerves [1, 2]. In addition, its disruption was known to enhance chemically-induced hepatic carcinogenesis [13]. Cx32 knockout (Cx32KO) mice showed a higher susceptibility to diethylnitrosamine (DEN)-induced carcinogenesis in the liver and lungs compared to wild-type mice [3, 9]. Thus, Cx32 is considered to be a tumor suppressor [24], although the molecular mechanisms remain to be elucidated. Meanwhile, there has been no report dealing with background lesions in Cx32KO mice in comparison with wild-type mice, the background strain of Cx32KO mice, during 24 months after birth. In the present study, we examined the incidence of neoplastic and non-neoplastic lesions in Cx32KO mice as well as those in wild-type mice.

Wild-type C57BL/6J mice were purchased from Charles River Laboratories (Yokohama, Japan). This strain is known to have a low susceptibility to spontaneous neoplastic lesions. Cx32KO mice were kindly provided by Willecke and colleagues [16], and then bred in our own facilities (Daiichi Sankyo Co., Ltd., Fukuroi, Japan). To confirm the genotype in Cx32KO mice, the excised tips of the tail were analyzed by a polymerase chain reaction (PCR). After identification, male and female Cx32KO and wild-type mice (50 mice/sex/strain) were housed until 24 months of age in polycarbonated cages (5 mice/cage) in a barrier-sustained room controlled at a temperature of 23 ± 1°C, relative humidity of 55 ± 5%, illumination time of 12 hr/day at an intensity of about 200 luces and ventilation of 10–15 cycles/hr. Basal diet (Certified Rodent Diet 5002: PMI Nutrition International, Inc., St. Louis, MO, U.S.A.) and fresh tap water were given *ad libitum*. At termination, all surviving mice were sacrificed under ether anesthesia, and were examined macro- and microscopically. The eyes were fixed in Bouin's fluid, and other organs and tissues were fixed in 10% buffered formalin. Then, the specimens were routinely prepared, stained with hematoxylin and eosin (HE stain) and examined with a light microscope. Animals found dead or sacrificed in extremis during the study were observed systemically. Results of histopathological examination were analyzed by Fisher's exact test, and were evaluated at a 5% significance level. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [8]. The experimental protocol was approved by the Animal Experimental Committee of the Daiichi Sankyo Co., Ltd., Japan.

During the 24-month observation period, the number of animals that died or were sacrificed in extremis was 1 male and 6 females for Cx32KO mice, and 8 females for wild-type mice, indicating no difference between the strains. The

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cause of these deaths may be at least in part due to poor body condition as a consequence of the development of spontaneous tumors such hemangiosarcoma, histiocytic sarcoma and malignant lymphoma at a background level in this strain [5].

In macroscopic and microscopic examinations, significant alterations were observed only in the liver, pituitary and kidney (Table 1).

In Cx32KO mice, hepatocellular carcinoma was found in 18% of the males and 4% of the females, and only the incidence for males was significantly higher under the present conditions (24 months old). Although hepatocellular adenomas in Cx32KO mice tended to be higher than those in wild-type mice, no significant difference in the incidence was noted. In C57BL/6 mice [14], the development of hepatocellular carcinomas was reported to be age-related, and its incidence increased gradually after 18 months of age. According to a previous report with Cx32KO mice [3], increased incidence of spontaneous hepatocellular carcinomas was not noted, because the animals were sacrificed at 18 months of age. Hepatocellular carcinomas in the present study were classified morphologically into trabecular pattern, and solid pattern with the eosinophilic or basophilic cytoplasm (Fig. 1), although it had not metastasized to other organs. These patterns resembled those in spontaneous neoplasms in B6C3F1 mice, as reported previously [5]. The difference in incidences of hepatocellular carcinoma between males and females may be explained on the basis of the sex hormones. Castration of male C57BL/6 × DS-F_1 mice results in a decrease in liver tumors, whereas the incidence of tumors increases following ovariectomy of females [22]. In male C57BL/6J and C57BL × C3H mice, testosterone was reported to enhance the liver tumor development, whereas ovarian hormones inhibited it [17, 19]. Recently, there has been increasing attention to the molecular mechanism underlying the carcinogenic potentials related to sex hormones. For example, androgen was recognized to induce/promote hepatocellular carcinomas via the androgen receptor with G1/S cell cycle progression [4]. In contrast, estrogen at a proper dose was known to protect hepatocytes from malignant transformation via downregulation of IL-6 release from Kupffer cells [15]. In Cx32KO mice given DEN, moreover, the incidence of hepatic tumors for males was reported to be higher than that for females, suggesting the involvement of sex hormones [3].

Certain hepatic-tumor promoting agents including phenobarbital, polychlorinated biphenyls, and dichlorodiphenyltrichloroethane were reported to inhibit GJIC and to increase proliferation of hepatocytes, which suggested that inhibition of GJIC was involved in tumor development [11]. In Cx32KO mice, which GJIC was depressed in the liver, cell proliferation accessed by incorporation of bromodeoxyuridine index was increasing in the hepatocytes [10, 18]. Moreover, decreased Cx32 expression has been observed in the early stage of pre-neoplastic lesions or hepatic tumors [13]. The development and growth of hepatocellular carcinoma were markedly accelerated in GJIC-inhibited transgenic rats carrying a dominant negative mutant of Cx32 and treated with DEN [7]. These reports suggested that disruption of Cx32/GJIC in the liver was closely related to hepatic tumor promotion and progression. On the other hand, connexin 26 (Cx26) was reported to co-localize with Cx32 in the liver, and hepatic Cx26 expression in Cx32KO mice was low compared to that in wild-type mice [16]. However, decreases in Cx26 expression were considered to be unrelated to the promotion of hepatic tumors, because administration of DEN to Cx26KO mice did not affect the onset of liver tumors [12]. Based on these observations, increased incidence of hepatocellular carcinoma in Cx32KO mice may be associated with enhanced tumor promotion and progression signals due to Cx32/GJIC deficiency.

In the pituitary, the incidence (4%) of adenomas in the pars distalis of female Cx32KO mice was significantly lower than that (20%) of female wild-type mice (Table 1). Generally, female mice are known to have higher sensitivity to development of spontaneous pituitary adenomas than male mice, probably indicating an involvement of sex hormones [6]. Additionally, there has been no report showing that Cx32 was expressed in the pituitary of rodents. Therefore, the decreased incidence of pituitary adenoma may not be a direct effect of Cx32 deficiency.

In non-neoplastic lesions, vacuolation in the renal tubular epithelium increased significantly in male Cx32KO mice compared to that of male wild-type mice. This was considered to be unrelated to Cx32 deficiency, because this finding occasionally occurs in male C57BL/6 mice [20]. According to previous studies using mice [20, 21], vacuolar changes in the renal tubular epithelium were more prominent for males than for females, although such alterations were not observed for male wild-type mice in our study. Thus, further studies are needed to elucidate these issues.

In conclusion, these results demonstrate that the incidence of hepatocellular carcinoma increases only in male Cx32KO mice, presumably due to enhanced tumor promotion and progression signals associated with Cx32 deficiency. These data will be useful as the background for future mechanistic studies for well known carcinogen using Cx32KO mice.

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Table 1. Spontaneous neoplastic and non-neoplastic lesions in wild-type and Cx32KO mice during a 24-month observation period

Lesions	Organ	Pathological findings	Males		Females	
			Wild-type	Cx32KO	Wild-type	Cx32KO
Neoplastic	Liver					
	MACRO	Nodule	4 (8)	14* (28)	2 (4)	10* (20)
	MICRO	Histiocytic sarcoma	6 (12)	7 (14)	3 (6)	7 (14)
		Hemangiosarcoma	1 (2)	2 (4)	0	1 (2)
		Hamangioma	3 (6)	2 (4)	1 (2)	1 (2)
		Malignant lymphoma	4 (8)	5 (10)	3 (6)	5 (10)
		Hepatocellular carcinoma	0	9** (18)	0	2 (4)
		Hepatocellular adenoma	0	4 (8)	1 (2)	5 (10)
		Mast cell tumor	0	0	1 (2)	1 (2)
	Pituitary					
	MACRO	Nodule	0	0	8 (16)	0*
	MICRO	Adenoma in pars distalis	0	0	10 (20)	2** (4)
		Adenoma in pars intermedia	0	0	0	1 (2)
		Carcinoma	0	0	1 (2)	0
Non-neoplastic	Liver					
	MACRO	Colored area	7 (14)	9 (18)	1 (2)	4 (8)
		Rough surface	1 (2)	0	2 (4)	2 (4)
		Atrophy	1 (2)	0	0	2 (4)
		Discoloration	1 (2)	1 (2)	0	1 (2)
		Cyst	0	4 (8)	1 (2)	2 (4)
		Enlargement	0	0	2 (4)	1 (2)
	MICRO	Increased granulopoiesis	2 (4)	1 (2)	0	1 (2)
		Inflammatory cell infiltration	1 (2)	0	0	0
		Thrombosis	1 (2)	0	0	0
		Proliferation of Kupffer cell	2 (4)	0	1 (2)	1 (2)
		Angiectasis	1 (2)	0	1 (2)	1 (2)
		Congestion	1 (2)	0	0	0
		Fatty change of hepatocyte, centrilobular	1 (2)	1 (2)	0	0
		Biliary cyst	0	3 (6)	0	2 (4)
		Eosinophilic foci	0	1 (2)	1 (2)	3 (6)
		Focal necrosis of hepatocyte	0	1 (2)	0	1 (2)
		Clear cell foci	0	0	1 (2)	0
		Vacuolated cell foci	0	0	0	1 (2)
		Eosinophilic cytoplasmic ateration of bile duct epithelium	0	0	1 (2)	0
	Pituitary					
	MACRO	Colored area	0	0	2 (4)	1 (2)
		Enlargement	0	0	1 (2)	0
	MICRO	Cyst	1 (2)	3 (6)	1 (2)	3 (6)
		Focal hyperplasia	0	1 (2)	0	1 (2)
		Cyst in pars distalis	0	0	1 (2)	0
		Focal hyperplasia in pars distalis	0	0	6 (12)	7 (14)
		Hyperplasia in pars intermedia	0	0	2 (4)	1 (2)
	Kidney					
	MACRO	Discoloration	1 (2)	0	1 (2)	1 (2)
		Cyst	2 (4)	0	1 (2)	1 (2)
		Enlargement	0	0	1 (2)	0
		Colored area	0	0	1 (2)	1 (2)
		Rough surface	0	0	0	1 (2)
	MICRO	Dilatation of renal pelvis	0	1 (2)	0	1 (2)
		Mineralization	0	2 (4)	0	0
		Cyst	2 (4)	3 (6)	2 (4)	2 (4)
		Osseous metaplasia	1 (2)	0	0	0
		Vacuolation of tubular epithelium	0	11** (22)	0	0
		Chronic nephropathy	5 (10)	5 (10)	9 (18)	10 (20)
		Glomerulopathy	0	0	2 (4)	0
		Hyperplasia of transitional epithelium	0	0	1 (2)	0

* $P<0.05$,** $P<0.01$ vs. the Wild-type group by the Fisher's exact probability test. Entries are the number of animals, including those that died or were sacrificed in extremis. Data in parentheses show incidence (% of animals) of each lesion. n=50. MACRO: Macroscopic examination. MICRO: Microscopic examination.

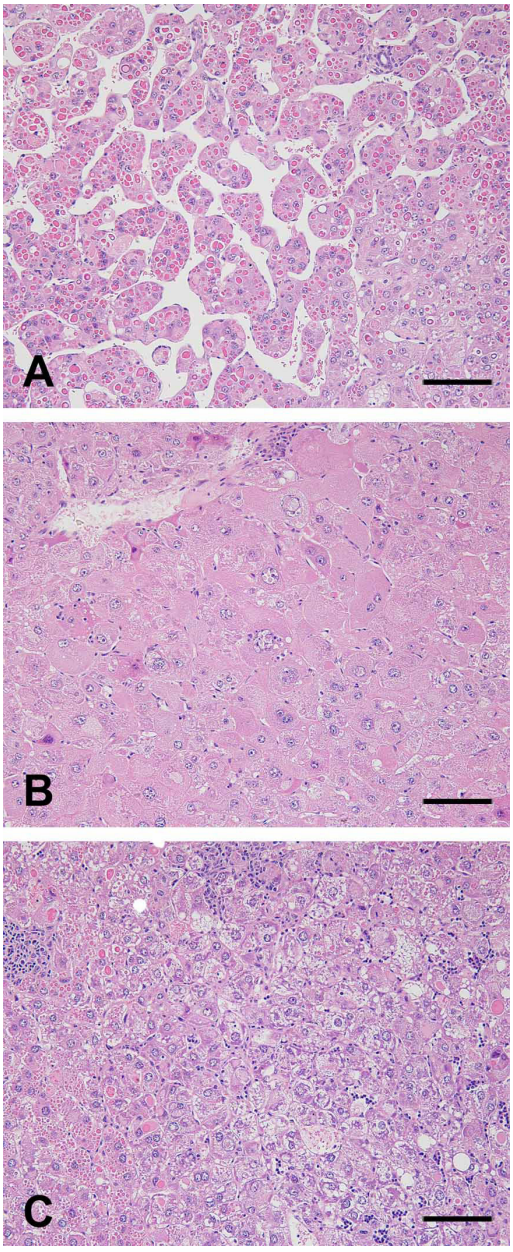

Fig. 1. Representative photomicrograph of hepatocellular carcinoma in a Cx32KO mouse at age 24 months. Note trabecular pattern (A), and solid pattern with eosinophilic (B) or basophilic (C) cytoplasm. Bar=100 μm. HE stain.

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