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Both Early and Late Stages of Hepatocarcinogenesis Are Enhanced in Cx32 Dominant Negative Mutant Transgenic Rats with Disrupted Gap Junctional Intercellular Communication

Naomi Hokaiwado · Makoto Asamoto · Mitsuru Futakuchi · Kumiko Ogawa · Satoru Takahashi · Tomoyuki Shirai

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Abstract Connexins are a family of transmembrane proteins essential for the gap junctions, which mediate cell-to-cell communication. Several connexins are reported to be tumor suppressors, and we have established transgenic (Tg) rats with a connexin 32 (Cx32) dominant negative mutant showing high sensitivity to early-stage diethylnitrosamine (DEN)-induced liver carcinogenesis. In this study, we carried out two independent experiments using Tg rats to further investigate the roles of disrupted Cx32 in late-stage carcinogenesis (carcinoma induction and metastasis) in the liver. In the first experiment, of 50 weeks' duration, DEN was administered at 6 weeks of age and at 26 weeks to explore the effects of carcinogen treatments at different stages. The number of hepatocellular carcinomas (HCCs) was significantly increased in Tg compared with non-Tg rats. The second experiment focused on the effects of Cx32 disruption on metastasis by HCCs induced by administration of DEN and *N*-nitrosomorpholine. Only Tg rats had multiple metastases of HCCs in the lung, and the development and growth of HCCs was dramatically accelerated in Tg compared to non-Tg rats. Thus, normal function of Cx32 may be essential for suppression of both early and late stages of hepatocarcinogenesis.

Keywords Gap junction · Connexin 32 · Transgenic rat · Liver · Tumor progression

N. Hokaiwado · M. Asamoto (✉) · M. Futakuchi · K. Ogawa · S. Takahashi · T. Shirai
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan
e-mail: masamoto@med-nagoya.cu.ac.jp

Introduction

Gap junctional intercellular communication (GJIC) plays important roles in tissue homeostasis (Oyamada, Oyamada & Takamatsu, 2005), cell differentiation (Mesnil et al., 2005; Trosko & Chang, 2001) and carcinogenesis (Mesnil et al., 2005; Trosko & Chang, 2001). Gap junctions themselves consist of two hemichannels called ''connexons,'' facing each other on cell surfaces and each comprising six connexin molecules (Willecke et al., 2002). Among over 20 members of the connexin family, connexin 32 (Cx32) and Cx26 are expressed in hepatic parenchymal cells (Mesnil et al., 2005; Oyamada et al., 2005; Willecke et al., 2002).

We previously reported our Cx32 dominant negative mutant transgenic rat bearing high copy numbers of the transgene to have decreased capacity for GJIC as measured by dye transfer *in vivo* (Asamoto et al., 2004). They also show resistance to hepatotoxicity of D-galactosamine and carbon tetrachloride (Asamoto et al., 2004) and high susceptibility in hepatocarcinogenesis induced by a single intraperitoneal (i.p.) administration of diethylnitrosamine (DEN, 200 mg/kg body weight) (Hokaiwado et al., 2005). Cx32 mutant transgenic (Tg) mice with a similar gene construct similarly demonstrate high susceptibility to chemically induced hepatocarcinogenesis (Dagli et al., 2004; Evert et al., 2002; Temme et al., 1997) and its promotion (Moennikes et al., 2000, 2003). The metastatic ability of hepatocellular carcinoma (HCC) has not yet been examined under GJIC-disrupted conditions in mice. However, GJIC is reported to play important roles in multistage hepatocarcinogenesis (Mesnil et al., 2005; Trosko & Ruch, 2002; Yamasaki et al., 1999), and several reports have suggested that restoration of connexin function might become a target of cancer therapy (King & Bertram, 2005;

Trosko & Ruch, 2002). In our previous experiment, Tg rats receiving a single DEN treatment developed significantly increased numbers and areas of preneoplastic lesions in their livers but tumor incidences were not significantly increased (Hokaiwado et al., 2005). The single administration of carcinogen at an early stage of hepatocarcinogenesis in the previous study may have been insufficient for tumor formation in Tg rats, and we hypothesized that additional carcinogen exposure at a later stage might increase cancer development. This hypothesis was tested in the present study, with an additional focus on the metastatic ability of induced HCC. In 1999, we introduced a new *in vivo* metastasis model in which HCC induced by sequential administration of DEN and *N*-nitrosomorpholine (NMOR) to rats frequently metastasizes to the lungs (Futakuchi et al., 1999; Yoshino et al., 2005). With this model, effects of Cx32 disruption on HCC metastasis formation in the lung were investigated.

Materials and Methods

Animals

The establishment, production and screening of Tg rats were described in detail previously (Asamoto et al., 2004).

Animals having high copy numbers (~50 copies) of the transgene were used in the present studies. Tg male rats were obtained by mating heterozygous Tg males and wild-type Sprague-Dawley (SD) females (Japan SLC, Shizuoka, Japan).

Experimental Protocols

Experiment 1

Male Tg and littermate wild-type (non-Tg) rats were each randomly divided into three groups. Group 1 was a control without carcinogen treatment (no treatment), group 2 received a single i.p. injection of 200 mg/kg body weight of DEN (Tokyo Kasei Kogyo, Tokyo, Japan) at the age of 6 weeks (DEN single) and the third group was given two i.p. injections of DEN 200 mg/kg body weight at the age of 6 weeks and 100 mg/kg body weight at the age of 26 weeks (DEN double). All animals were killed at the age of 56 weeks.

Experiment 2

The fundamental protocol for induction of metastatic liver tumors in the lung was reported previously (Yoshino et al., 2005). Male 6-week-old Tg and non-Tg rats were given a single i.p. injection of DEN at a dose of 100 mg/kg body weight, and then, after a 3-day interval, they received 40 ppm NMOR (Tokyo Kasei Kogyo) in drinking water for 14 weeks; after a further 14-week observation period, all animals were killed.

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University, Graduate School of Medical Sciences. The rats were randomly divided into groups of three animals per plastic cage with hard wood chips as bedding and maintained in an air-conditioned room under specific pathogen free conditions at 22 ± 2°C and 55 ± 5% humidity with a 12-h light/dark cycle. Food (Oriental MF; Oriental Yeast, Tokyo, Japan) and drinking water were available *ad libitum* throughout.

Immediately after death, the livers were excised, weighed, cut into slices of 2–3 mm thickness, fixed in 10% buffered formalin and routinely processed for embedding in paraffin for histological evaluation. Sections were stained with hematoxylin and eosin. In experiment 2, the lungs were inflated with 10% buffered formalin injected through the trachea and separated into three right lobes and one left lobe. In total, eight to 13 slices were made for each rat, processed for production of paraffin sections and stained with hematoxylin and eosin.

Statistical Analysis

Statistical analysis was performed with Fisher's exact probability test for the incidence of liver tumors in experiments 1 and 2. Analysis of variance (ANOVA) followed by the *post hoc* Bonfferoni/Dunn test for the remaining data in experiment 1 and Student's *t*-test for the data in experiment 2 were also employed with the StatView (Berkeley, CA) J 5.0 program.

Results

Experiment 1

Double treatment with DEN suppressed body weight gain of both Tg and non-Tg rats compared with the single DEN and no treatment groups, but the differences were not statistically significant. However, relative liver weights of Tg rats receiving double DEN were significantly heavier than those of Tg rats receiving single DEN or no treatment as well as those of non-Tg rats with double DEN (Table 1). Many neoplasms occurred in the livers of each DEN treatment group (Table 1). Generally, tumors were well to moderately differentiated HCCs (Fig. 1d). Clear intergroup histological differences were not found. However, some

Table 1 Body and relative liver weights and multiplicity of adenomas and HCCs (Experiment 1)

		No. of rats	Weights		Incidence [a]	Multiplicity (No./animal)	
			Body (g)	Relative liver (%)	(%)	Adenoma	HCC
Non-Tg	No Treatment	12	628.6±39.4	2.83±0.26	0/12 (0)	0	0
	DEN Single	11	627.4±47.7	3.22±0.19	5/11 (46)	0.55±1.04	0.36±0.67
	DEN Double	12	571.0±90.5	3.10±0.38	10/12 (83)	1.67±2.15	1.33±1.44
Tg	No Treatment	12	627.8±69.9	2.62±0.19	0/12 (0)	0	0
	DEN Single	12	607.9±42.7	3.12±0.21	9/11 (75)	0.67±0.78	1.00±1.21
	DEN Double	10	583.1±66.2	4.07±1.45 *	10/10 (100)	4.90±8.60 *	5.20±4.16 *

* ANOVA followed by Bonferroni/Dunn *post hoc* test, $P < 0.05$

[a] Indicates number of rats bearing tumor (both adenoma and HCC)

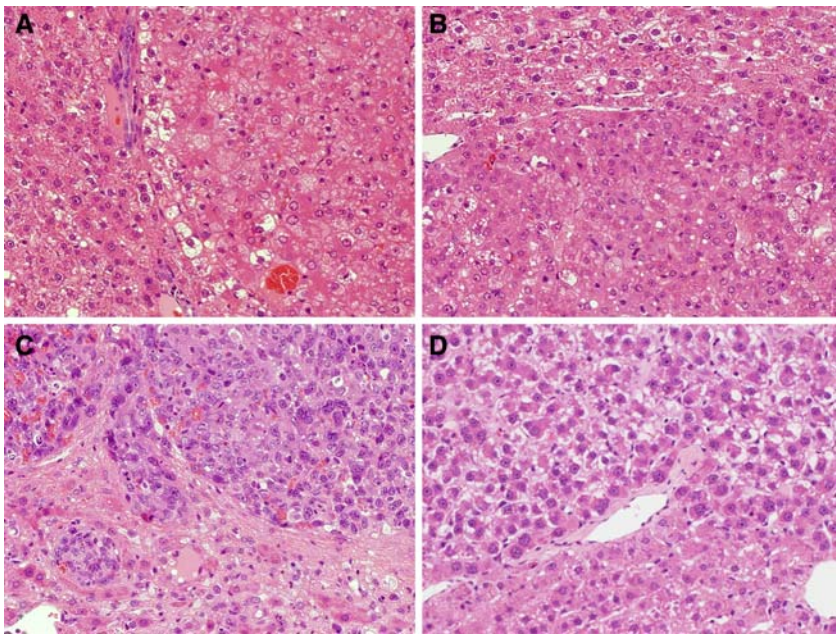

Fig. 1 Representative adenomas (**a,b**) and HCCs (**c,d**) induced by double DEN treatment in Tg and non-Tg livers. Clear histological differences were not observed between Tg (**a,c**) and non-Tg (**b,d**) rats, except with a few more aggressive HCCs in the Tg DEN double treatment group (**c**)

HCCs in Tg rats with double DEN demonstrated a poor differentiated histology, with atypical basophilic cells and nuclei atypia, as shown in Figure 1c. The multiplicity of HCCs in Tg rats with double DEN was significantly higher than that in the other groups. Tg rats also showed higher incidences of adenomas and HCCs with each DEN treatment than their non-Tg counterparts, but this was not statistically significant (Table 1).

Experiment 2

About 10 weeks after the end of the NMOR treatment (week 24), the first Tg rat died, and a total of four Tg rats were lost before the end of the study (these cases were excluded from the data). The causes of sudden death were massive bleeding into the peritoneal cavity from liver tumors. All these rats had lung metastases. The final body

Table 2 Body and relative liver weights and incidence of tumors (Experiment 2)

	Number of rats	NMOR	Weight		Incidence (%)	
			Body (g)	Relative liver (%)	HCC	Metastasis[a,b]
Non-Tg	15	+	504.3 ± 45.9	4.66 ± 0.79	100	0
Tg	10	+	458.1 ± 17.3**	10.61 ± 5.25*	100	60

*$P < 0.05$, **$P < 0.001$, Student's *t*-test

[a] HCC metastasis in lung

[b] $P < 0.01$, Fisher's test

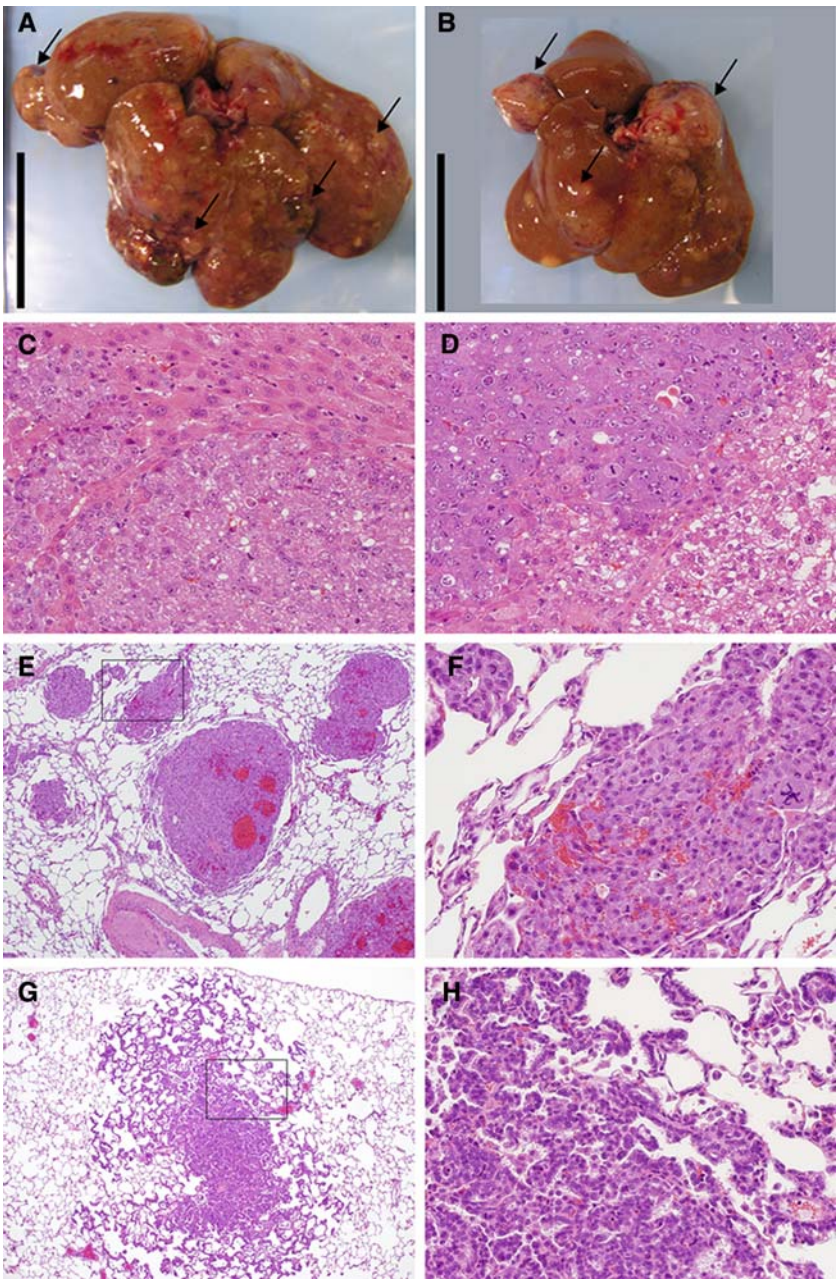

Fig. 2 Representative photographs of lesions obtained in experiment 2. Macroscopic findings with multiple tumors (*arrows*) apparent on the surfaces of both Tg (**a**) and non-Tg (**b**) livers. However, the Tg rat has more tumors. Bar = 5 cm. Representative liver cancers are illustrated in Tg and non-Tg cases in **c** and **d**, respectively. Lung multiple metastases are evident in the Tg case (**e**, ×4 objective; **f**, ×20 objective). **g, h** Alveolar epithelium hyperplasia in a non-Tg lung (**g**, ×4 objective; **h**, ×20 objective)

weights of Tg rats were extremely low compared with those of the non-Tg rats (Table 2), and the liver weights were significantly higher (Table 2, Fig. 2a,b). Both Tg and non-Tg rats had moderately differentiated HCCs without apparent histological differences (Table 2, Fig. 2c,d). However, metastasis of HCCs to the lung was found in

only the Tg rats, at 60% incidence (Table 2, Fig. 2e,f). Alveolar cell hyperplasias (Fig. 2g,h) were also found in the lungs of Tg and non-Tg rats, at incidences of 20% and 47%, respectively.

Discussion

The present study clearly showed that our Tg rats are more susceptible to both early- and late-stage administration of DEN than non-Tg littermates and that HCCs induced in Tg rats have an elevated propensity for metastasis. Generally, most carcinogen-induced, enzyme-altered foci in rat liver show markedly lowered GJIC and low numbers of Cx32-positive spots (Krutovskikh, Oyamada & Yamasaki, 1991; Mesnil et al., 2005; Sakamoto et al., 1992). Furthermore, progressive decrease of Cx32 expression has been observed from early preneoplasia to HCC (Mesnil et al., 2005; Tsuda et al., 1995), suggesting that disruption of GJIC is of advantage for every step of hepatocarcinogenesis.

The *in vivo* lung metastasis model of rat HCC established by our group (Futakuchi et al., 1999; Yoshino et al., 2005) is useful for investigation of metastasis and its modification by exogenous chemicals. In the present study, no lung metastasis was found in the non-Tg rats, probably because of strain differences (SD *vs.* F344). However, lung metastasis was frequently present in Tg rats, indicating that growth of HCCs in animals with disrupted Cx32 is aggressive, despite no clear differences in histology. However, the likelihood of metastasis may have been raised by the greater number of tumors induced; to clarify the issue of whether metastasis increased due to a malignant phenotype, further molecular investigations are needed.

King & Lampe (2004) reported that Cx32-deficient mice showed a higher sensitivity to DEN-induced lung carcinogenesis. In the present work, however, only hyperplastic alveolar lesions were induced in both Tg and non-Tg rats, without any significant difference. The reason for the variation in sensitivity to lung tumorigenesis between mice and rats is unclear, but one possibility is that Cx32 disruption in our rat model occurs in the liver but not the lung.

It is well known that Cx26 is colocalized with Cx32 in the liver. However, liver tissue of our Tg rats did not show positive Cx26 signals on immunostaining (Asamoto et al., 2004), resembling Cx32-deficient mice (Nelles et al., 1996). Cx26 itself is reported to have tumor-suppressor functions in other organs (Lee et al., 2002), but whether there are specific contributions of loss of Cx26 with disruption of Cx32 is not clear. To elucidate the roles of Cx26 and interaction with Cx32 during hepatocarcinogenesis, further investigations are needed.

In conclusion, our present data demonstrated that disruption of Cx32 in the liver promotes both early and late stages of hepatocarcinogenesis, with probable important roles for connexins in both lesion induction and progression of malignancy.

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