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Original

Promoting Effects of Streptozotocin-induced Diabetes on Induction of Hepatic Preneoplastic Lesions by Diethylnitrosamine in Rats

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Abstract: The effects of streptozotocin (STZ)-induced diabetes on induction of hepatic preneoplastic lesions by diethylnitrosamine (DEN) were investigated in male Fischer rats. A single dose of STZ was injected intravenously either 2 weeks before or after initiation with DEN. The blood glucose levels were significantly elevated from 1 week after STZ-injection until autopsy. The numbers of GST-P positive foci at 1 week after DEN administration in the STZ-injected rats were similar to those in the non-diabetic rats. In contrast, both the numbers and areas of GST-P positive foci > 2 mm in diameter 8 weeks after DEN administration were increased significantly in the rats treated with STZ after DEN exposure compared with the non-diabetic control rats. The results suggest that hepatic preneoplastic lesions initiated with DEN are promoted by STZ treatment-inducing diabetes. (J Toxicol Pathol 2010; **23**: 125–131)

Key words: streptozotocin, diabetes, diethylnitrosamine, liver, GST-P positive foci, hepatocarcinogenesis

Introduction

In recent years, epidemiological surveys have revealed that diabetic patients are at high risk of developing a variety of cancers[1–4]. In particular for hepatocellular carcinoma (HCC), diabetic individuals have a 2-4-fold elevated risk as compared with their non-diabetic counterparts[1–3, 5–10]. It is considered that diabetes could be an independent risk factor for HCC, separate from viral hepatitis and heavy alcohol consumption[5,8,10]. Although elevation of the fasting serum glucose level[4] or insulin[11] has been suggested to be involved in the underlying mechanisms, this has yet to be clarified in detail.

Experimental studies on the effects of diabetes on hepatocarcinogenesis have been limited. There are two types of animal model of diabetes, a spontaneous one and a drug-induced one. The streptozotocin (STZ)-induced diabetic rat model is widely used[12–14] as a model of Type-1 diabetes, because STZ reduces insulin secretion by selectively destroying pancreatic beta cells[15]. In the present work, we investigated the effect of diabetes due to STZ on diethylnitrosamine (DEN)-induction of rat hepatocarcinogenesis.

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Materials and Methods

Animals

Male F344/Ducrj (Fischer) rats were obtained at 4 or 5 weeks of age from Charles River Laboratories Japan Inc. (Atsugi, Japan) and used for the experiment after quarantine and acclimation for 1 week. The animals were individually housed in stainless steel wire-mesh cages in an air conditioned room (room temperature 23 ± 2°C, relative humidity 55 ± 10%, ventilation 10–20 times/h, and a 12 light/12 dark cycle). During the entire period of study, the rats were supplied with pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*.

All experimental procedures were performed in accordance with the Law for the Humane Treatment and Management of Animals (Law No.105, 1973), "Basic policies for the conduct of animal experiments in research institutions under the jurisdiction of the Ministry of Health, Labor and Wel-

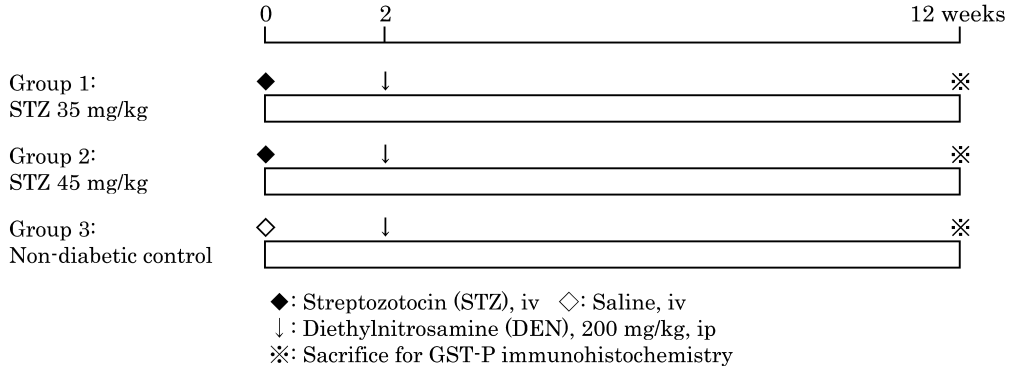

Fig. 1. Design of Experiment 1.

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Chemicals and preparation

STZ, obtained from Sigma Chemical Co., USA, was dissolved in 0.05 M citrate buffer solution (pH: 4.5)[16]. DEN, obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan, was diluted to 50 mg/ml in saline for use[17].

Experiment protocols

Experiment 1: In experiment 1, the effects of STZ-induced diabetes on the induction of hepatic preneoplastic lesions by DEN were investigated (Fig. 1). Five-week-old rats were randomly divided into three groups comprised of 15 rats/group. The first and second groups were injected with a single dose of STZ (35 mg/kg or 45 mg/kg in 0.05 M citrate buffer) into the tail vein at 1 ml/kg at a speed of 0.15 ml/min for induction of diabetes[18]. The third group was given the vehicle in the same manner. Two weeks later, all rats were injected with a single dose of DEN at a dose of 200 mg/kg b.w. intraperitoneally at 4 ml/kg to initiate hepatocarcinogenesis. At week 12, all rats were euthanized under ether anesthesia.

Experiment 2: In experiment 2, the effects of STZ-induced diabetes particularly on the initiation step of hepatocarcinogenesis were investigated by stopping the experiment at 1 week after DEN exposure (Fig. 2).

Five-week-old rats were randomly divided into two groups. The 16 rats comprising the first group were injected with a single dose of STZ (45 mg/kg in 0.05 M citrate buffer), whereas the 10 rats comprising the second group were given the vehicle in the same manner as Experiment 1. Two weeks later, all rats were injected with a single dose of DEN (200 mg/kg) intraperitoneally, and 1 week later (week 3), all rats were euthanized under ether anesthesia.

Experiment 3: The effects of STZ-induced diabetes on the promotion stage of hepatocarcinogenesis resulting from administration of DEN were investigated using a medium-term rat liver bioassay for carcinogens[17].

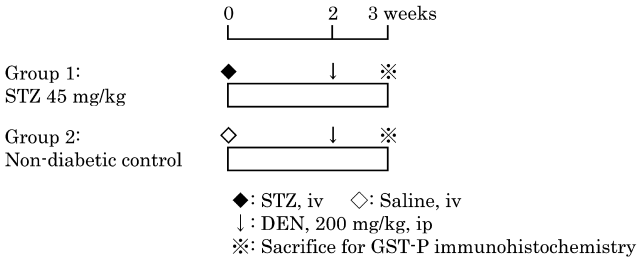

Fig. 2. Design of Experiment 2.

Six-week-old rats were randomly divided into four groups comprised of 15 rats/group. All rats were injected with a single dose of DEN (200 mg/kg) intraperitoneally. Two weeks later, the first and the second groups were injected with a single dose of STZ (15 mg/kg or 30 mg/kg in 0.05 M citrate buffer) into the tail vein in the proportion of 1 ml/kg at a speed of 0.15 ml/min, which induced diabetes (Fig. 3). The third and the forth groups were given the vehicle. The forth group was fed powder diet containing 0.05% phenobarbital for the following 6 weeks as a positive control for promotion of hepatocarcinogenesis.

At week 3, all rats were subjected to two-thirds partial hepatectomy to accelerate the carcinogenic process. At week 8, all rats were euthanized under ether anesthesia (Fig. 3).

Experiment 4: The effects of STZ-induced diabetes on the promotion step of hepatocarcinogenesis by resulting from administration of DEN without partial hepatectomy were investigated.

Six-week-old rats were randomly divided into two groups comprising 15 and 16 rats. All rats were injected with a single dose of DEN (200 mg/kg) intraperitoneally. Two weeks later, the first group was injected with a single dose of STZ (30 mg/kg in 0.05 M citrate buffer), whereas the second group was given the vehicle. At week 10, all rats were euthanized under ether anesthesia (Fig. 4).

Measurement of blood glucose

One or 2 weeks after STZ administration and at necropsy, blood samples were collected from the tail vein.

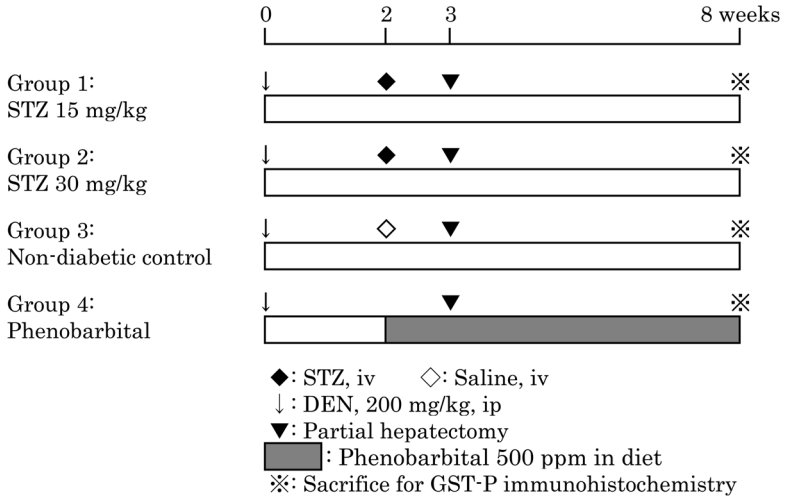

Fig. 3. Design of Experiment 3.

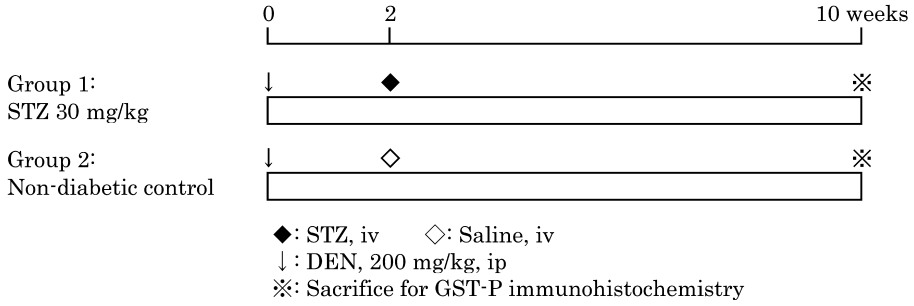

Fig. 4. Design of Experiment 4.

The plasma glucose concentration was measured by the glucose oxidase method using a Beckman glucose analyzer II (Beckman Instruments, Fullerton, CA, USA).

Immunohistochemical examination of the glutathione S-transferase placental form (GST-P)

Soon after sacrifice of the rats, livers were promptly excised and weighed, and sections 3–4 mm thick were cut from the right posterior lobe and fixed in ice-cold acetone for immunohistochemical examination of GST-P. The avidin-biotin complex method (Vectastain Elite Kit, Vector Laboratories Inc.) was used to demonstrate GST-P positive cell foci using a specific antibody (MBL, Nagoya, Japan).

The numbers and areas of GST-P positive foci were measured quantitatively using the multipurpose image processor IPAP-WIN (Sumika Technoservice Corporation, Hyogo, Japan).

Statistical analysis

Blood glucose, final body weight, liver weight and the data expressed as numbers and areas of GST-P positive foci per unit area of the liver slices were assessed by comparing the values between groups. Statistical analyses were carried out using the Student's *t*-test.

Results

Experiment 1

Blood glucose levels in STZ-injected groups were STZ dose-dependently and significantly elevated from 2 weeks after the STZ treatment until the end of the experiment (Table 1). The body weights of the STZ-injected groups, particularly in the 45 mg/kg STZ-injected group, were consistently lower than those of the non-diabetic control group. The relative liver weights of the STZ-injected groups also showed significant increases when compared with the non-diabetic control group. Both the numbers and areas of GST-P positive foci > 2 mm in diameter were increased STZ dose-dependently and significantly in the STZ-injected groups when compared with the non-diabetic control group (Table 1, Fig. 5). In particular the 45 mg/kg STZ-injected group showed a fivefold or sixfold increase when compared with the non-diabetic control group (Table1).

Experiment 2

In the 45 mg/kg STZ-injected group, significant elevation of the blood glucose level and significant reduction of body weight gain were observed. The absolute and relative liver weights of the STZ-injected group showed significant

Table 1. Blood Glucose Levels, Body and Liver Weights, and Quantitative Values of GST-P Positive Foci in Experiment 1

Group	Treatment	No. of rats	Blood glucose (mg/dL)		Final body weight (g)	Liver weight		GST-P positive foci	
			Week 2	Week 12		Absolute (g)	Relative (%)	No./cm^2	Area (mm^2)/cm^2
1	35 mg/kg STZ +DEN	15	339 ±129***	316 ± 139***	205.2 ± 28.0***	8.0 ± 0.6*	4.0 ± 0.6***	3.0 ± 1.9**	0.22 ± 0.18**
2	45 mg/kg STZ +DEN	15	503 ±77***	450 ± 35***	152.6 ± 25.1***	7.3 ± 0.7	4.9 ± 0.7***	5.2 ± 2.2***	0.31 ± 0.16***
3	DEN control	15	127 ± 6	108 ± 2	251.2 ± 13.6	7.5 ± 0.4	3.0 ± 0.1	1.0 ± 1.0	0.05 ± 0.06

Values are means ± SD. *,**,*** Significantly different from the DEN control group values at p <0.05, p <0.01, p <0.001, respectively.

increases when compared with the control group.

There was no significant difference in the numbers of GST-P-positive foci per unit area between the STZ-injected group and the non-diabetic control group (data not shown).

Experiment 3

During the experiment, 4 normal control rats, 3 of 15 mg/kg STZ-injected rats, 7 of 30 mg/kg STZ-injected rats and 4 phenobarbital administered rats died from surgical error or post-surgical debilitation. These dead rats were excluded from the effective numbers in the experimental results.

There was no difference in the blood glucose levels between the 15 mg/kg STZ-injected group and the normal control group (Table 2). However, significantly elevated blood glucose was observed in the 30 mg/kg STZ-injected group. Each STZ-injected group showed similar changes of body weight as in the normal control group throughout the experimental period. There were no differences in the liver weights between the 15 mg/kg STZ-injected group and the normal control group. However, significant increases of both absolute and relative liver weights were observed in the 30 mg/kg STZ-injected group and the phenobarbital administered group (Table 2).

The numbers and areas of GST-P positive foci in the STZ-injected groups showed a tendency to increase, but did not significantly differ from the control values (Table 2). In the phenobarbital group, the numbers and areas of GST-P positive foci showed threefold and fourfold increases, respectively, when compared with the controls (Table 2).

Experiment 4

In the STZ-injected group, significantly elevated blood glucose and reduced body weight gain were observed (Table 3). The relative liver weights of the STZ-injected group were twofold higher than the non-diabetic control values. Six of the STZ-injected rats showed discoloration of the liver, indicating deposition of fat.

Both the numbers and areas of GST-P positive foci in the STZ-injected group showed threefold and fourfold increases respectively, when compared with the non-diabetic control values (Table 3).

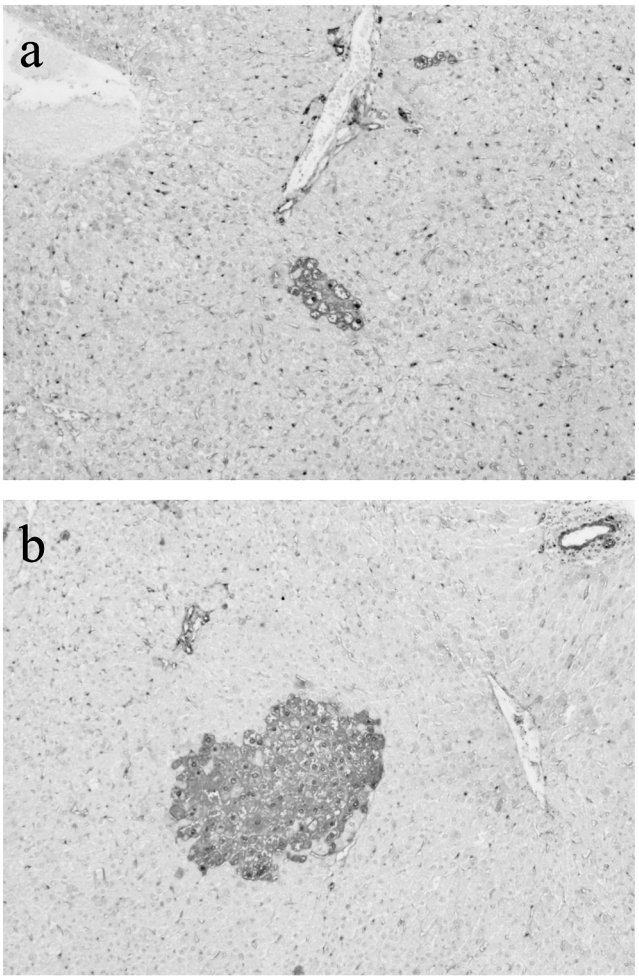

Fig. 5. Representative GST-P foci in the DEN control (a) and DEN groups receiving 45 mg/kg STZ (b) in Experiment 1.

Discussion

In the present study, STZ treatment steadily increased the blood glucose levels throughout the experimental period, pointing to induction of a continuous state of diabetes. Furthermore, development of GST-P positive foci, well established rat hepatic preneoplastic lesions, was enhanced in the diabetic rats and was STZ-dose dependent.

Examination of whether the increase of hepatic preneoplastic lesions in STZ-induced diabetic rats was attributable to accelerated initiation by DEN or promotion of the growth of initiated preneoplastic lesions, suggested that the latter was the more important, because the numbers of GST-P positive foci 1 week after DEN administration (Fig. 2) in the

Table 2. Blood Glucose Levels, Body and Liver Weights and Quantitative Values of GST-P Positive Foci in Experiment 3

Group	Treatment	No. of rats	Blood glucose (mg/dL)		Final body weight (g)	Liver weight		GST-P positive foci	
			Week 3	Week 8		Absolute (g)	Relative (%)	No./cm^2	Area (mm^2)/cm^2
1	DEN+15mg/kg STZ	12	129 ± 5	116 ± 3	234.7 ± 14.0	7.3 ± 0.8	3.1 ± 0.2	5.0 ± 3.1	0.36 ± 0.26
2	DEN+30mg/kg STZ	8	377 ± 75***	219 ± 136**	228.9 ± 20.1	8.1 ± 0.6**	3.6 ± 0.5**	5.9 ± 2.6	0.44 ± 0.29
3	DEN control	11	123 ± 8	114 ± 6	224.7 ± 15.2	6.9 ± 0.8	3.1 ± 0.2	4.2 ± 2.5	0.27 ± 0.21
4	DEN+PB	11	–	–	243.7 ± 9.9**	9.4 ± 0.5***	3.9 ± 0.1***	15.3 ± 4.0***	1.05 ± 0.39***

Values are means ± SD. **,*** Significantly different from the DEN control group values at $p < 0.01$, $p < 0.001$, respectively.

Table 3. Blood Glucose Levels, Body and Liver Weights, and Quantitative Values of GST-P Positive Foci in Experiment 4

Group	Treatment	No. of rats	Blood glucose (mg/dL)		Final body weight (g)	Liver weight		GST-P positive foci	
			Week 3	Week 10		Absolute (g)	Relative (%)	No./cm^2	Area (mm^2)/cm^2
1	DEN+STZ 30 mg/kg	15	362 ± 46***	410 ± 101***	133.6 ± 25.8***	8.4 ± 1.5	6.6 ± 2.0***	8.3 ± 3.7***	0.66 ± 0.43***
2	DEN control	15	81 ± 6	77 ± 5	247.6 ± 14.1	7.6 ± 0.5	3.1 ± 0.1	2.5 ± 1.8	0.17 ± 0.15

Values are means ± SD. *** Significantly different from the DEN control group values at $p < 0.001$.

STZ-injected group were thus similar to those in the non-diabetic control group. We then utilized the medium-term rat liver bioassay for carcinogens to examine the promotion potential of STZ-induced diabetes. Development of GST-P positive foci was only slightly enhanced in the STZ-injected groups, without statistical significance. The decrease of the blood glucose levels in diabetic rats after partial hepatectomy was thought to be the cause of this result. Therefore, we conducted an additional experiment without partial hepatectomy to reexamine the promotion effect of STZ-induced diabetes, in which significant increases of the numbers and areas of GST-P positive foci were observed in the STZ-injected group. These data clearly indicated that STZ-induced diabetes promotes development of hepatic preneoplastic lesions in rats.

Saha *et al*.[19] previously reported that the total number and size of hepatic hyperplastic nodules was increased by STZ administration either before or after initiation with DEN. Furthermore, both the hepatic GST and microsomal GGT activities were significantly increased in these STZ administered rats. Because increases of these biomarkers were more pronounced in rats given STZ after DEN initiation, they proposed that the diabetic condition in rats accelerates DEN-induced hepatocarcinogenesis. Our results are in agreement. STZ-induced diabetic rats have been widely used as a type 1 diabetic model because STZ selectively destroys beta cells in the pancreatic islands, resulting in reduction of insulin secretion. Together with the data of Saha *et al*.[19], our findings clearly demonstrate that high blood glucose conditions in diabetic rats enhance liver carcinogenesis after exposure to a carcinogenic substance.

In humans, type 2 diabetes accounts for the majority of all diagnosed cases of diabetes. The level of insulin is typically high during the development and early stages of type 2 diabetes. Activation of the insulin receptor by its ligand, or cross-activation of the insulin-like growth factor 1 receptor, has been shown to be mitogenic and promote tumorigenesis[20]. Oshima *et al*. investigated the effect of insulin on liver tumorigenesis by DEN in STZ-induced diabetic rats using a medium-term rat liver bioassay for carcinogens. Significant increase in the area of hyperplastic liver nodules was the outcome[21]. Dombrowski *et al*. demonstrated that islet transplantation into the livers of STZ diabetic rats can induce hepatocellular preneoplastic lesions and neoplasms at a high incidence after 22 months[22]. They also showed that islet transplantation into the livers of BB/Pfd rats, which develop spontaneous autoimmune diabetes similar to human type 1 diabetes, caused hepatocellular neoplasia[23]. These results demonstrated that combined hyperinsulinism and hyperglycemia may act as a carcinogenic factor for the development of HCCs in diabetic rats.

STZ is known as a genotoxic chemical in rats[24]. Dombrowski *et al*. confirmed that hepatocarcinogenesis in their STZ diabetic model is independent from STZ[22,23]. In the present study, STZ did not accelerate initiation by DEN, and preneoplastic lesions were not enhanced significantly by STZ injection with partial hepatectomy. Therefore, the increase of the preneoplastic lesions in the STZ-injected group without partial hepatectomy may be independent from the genotoxicity of STZ. We hypothesized that hyperglycemia-mediated oxidative stress promoted preneoplastic lesions.

In recent years, non-alcoholic steatohepatitis (NASH) has received a great deal of attention as a nonviral cause of hepatocarcinoma in humans[25–27]. It is known that 25% of NASH cases progress to cirrhosis through 10 years of follow-up[28], and the affected individuals may develop liver cancer[29,30]. NASH is considered to be caused by fatty deposits in the liver facilitated by an increase of insulin resistance and is likely associated with additional hepatocyte damage

by oxidant stress[31]. Many patients with NASH have hyperinsulinemia linked to insulin resistance[32,33]. Furthermore, oxidant stress derived from hyperglycemia in diabetic patients aggravates insulin resistance and increases inflammatory cytokines in the blood[34,35]. Therefore, it is suggested that hyperinsulinemia and hyperglycemia play a role in the progression of hepatocellular carcinoma. The STZ-induced diabetic rats in our experiment showed increases of liver weight and discoloration (fatty deposition), but similarities to the pathological conditions of NASH and the involvement of oxidant stress remain to be ascertained.

In conclusion, the present experiemental results demonstrated that hepatic preneoplastic lesions initiated with DEN are promoted by STZ-treatment inducing diabetes. This might be the case in type 2 diabetic patients, and our experimental animal model might thus be of general use to study the mechanisms underlying elevated hepatocarcinoma risk in diabetic patients.

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