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Original article

Preventive effect of JTE-522, a selective cyclooxygenase-2 inhibitor, on DEN-induced hepatocarcinogenesis in rats

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ABSTRACT

Background. – Chemopreventive effect of a selective cyclooxygenase-2 (COX-2) inhibitor JTE-522 on diethylnitrosamine (DEN)-induced hepatocarcinogenesis was evaluated in Wistar rats.
Methods. – Animals in the control group (G1) were injected with phosphate buffered saline (PBS), those in hepatocellular carcinoma (HCC) group (G2) were injected with DEN with regular foods for 14 weeks, and those in the treatment groups were injected with DEN for 14 weeks fed with JTE-522 for 7 (G3) and 14 weeks (G4), respectively. Proliferation and precancerous lesions were evaluated by expression levels of proliferating cell nuclear antigen (PCNA) and glutathione S-transferase-P (GST-P), respectively by immunohistochemistry and Western blot analysis. Apoptosis and oxidative stress were evaluated by TdT-mediated dUTP-biotin nick-end labeling (TUNEL) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) staining, respectively.
Results. – After 14 weeks of the treatment, HCC was developed in G2, G3, and G4 showing no significant differences in gross appearance and histology of the liver among the three groups. There were no significant differences in the expression levels of PCNA and numbers of TUNEL and 8-OHdG positive cells in the liver among the three groups. However, GST-P positive area was significantly suppressed in G3 and G4 compared to G2.
Conclusion. – Our data revealed that JTE-522 had a modest inhibitory effect on hepatocarcinogenesis in rats in a manner independent of induction of apoptosis and inhibition of oxidative stress.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and the third-leading cause of death from cancer worldwide [1]. Most patients with HCC are complicated with persistent hepatitis B and C infections. Although ablative therapies including ethanol injection and radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE), and surgical resection have been applied to patients with HCC, curative treatments are hampered by frequent recurrence of HCC in the remnant liver [2]. Although systemic chemotherapy has been challenged to patients with advanced stages of HCC, it is mostly ineffective [3]. Given the poor outcome of patients with HCC, the exact molecular mechanisms of hepatocarcinogenesis have been intensively investigated, however, effective therapeutic compounds targeting key molecules involved in hepatocarcinogenesis have not been discovered yet.

Cyclooxygenase-2 (COX-2) is an inducible enzyme converting arachidonic acid to prostaglandins (PGs). Several lines of evidence suggest that COX-2 may play a pivotal role in hepatocarcinogenesis. For example, COX-2 is overexpressed in HCC [4], forced expression of COX-2 in HCC cells promotes the cellular growth [5], selective COX-2 inhibitors suppress the proliferation of HCC cells [6–9], and selective COX-2 inhibitors prevent hepatocarcinogenesis in rodents [10]. However, the exact molecular mechanisms by which selective COX-2 inhibitors suppress the growth of HCC remain unsolved.

Diethylnitrosamine (DEN) is a powerful hepatocarcinogen which has been used as an initiating agent in two-stage (initiation and promotion) protocols for hepatocarcinogenic studies. DEN is metabolized to reactive electrophilic reactants, alter the structure of DNA, and form alkyl DNA adducts, inducing chromosomal aberrations and micronuclei in the rat liver [11].

Among glutathione S-transferases (GSTs), a family of detoxification enzymes which catalyze the conjugation of glutathione with a large number of carcinogens, placental GST (GST-P) is specifically expressed during rat hepatocarcinogenesis, and has been used as a reliable tumor marker for experimental hepatocarcinogenesis in the rat [12].

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In the present study, we evaluated the preventive effects of JTE-522, a selective COX-2 inhibitor, on the development of DEN-induced HCC in rats. Our data revealed that JTE-522 might have inhibitory effects on hepatocarcinogenesis in rats in a manner independent of induction of apoptosis and inhibition of oxidative stress, although such effects appeared to be moderate.

2. Materials and methods

2.1. Chemicals

JTE-522, a selective cyclooxygenase-2, was kindly provided by Japan Tobacco, Inc. (Tokyo, Japan) and dissolved in ethanol. DEN and an anti-β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO, USA). Pentobarbital was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Antiproliferating cell nuclear antigen (PCNA), Bcl-2, and BAX antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-GST-P antibody was purchased from Assay Designs, Inc. (Ann Arbor, MI, USA). An anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody was purchased from the Japan Institute for The Control of Aging, Nikken SEIL Co. (Shizuoka, Japan). Secondary antimouse and antirabbit horseradish peroxidase antibodies were obtained from GE Healthcare Ltd. (Buckinghamshire, UK). All other chemicals and solvents used in this study were of analytical grade.

2.2. Experimental animals, treatments, and tissue collection

Male Wistar rats weighing ~200 g were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). All animals received humane care and protocols were approved by the Tottori University Animal Ethics Committee. The animals were randomized, divided into four groups (n = 10 in each group, Fig. 1), and housed two per cage with rice husks for bedding in an air-ventilated room under a 12-h light/dark cycle with constant temperature (22 °C) and humidity (55%). The animals were allowed free access to food and tap water *ad libitum* during the experiment. Animals in group 1 (G1) (untreated control) were intraperitoneally injected with 300 μl of phosphate buffered saline (PBS) weekly for 14 weeks. Animals in group 2 (G2), group 3 (G3), and group 4 (G4) were intraperitoneally injected with DEN (50 mg/kg body weight) in PBS weekly for 14 weeks. In G3 and G4, the rats were fed with JTE-522 in powder form (30 mg/kg body weight per day) by daily gavage for 7 and 14 weeks, respectively (Fig. 1). Body weights, food consumption, and water intake were monitored weekly throughout the experimental period. After 14 weeks of the treatments, animals were killed under anesthesia by pentobarbital. Blood samples were withdrawn via cardiac puncture and serum samples were stored at −30 °C until analysis. Immediately after the livers were excised, they were divided into two sections for histological examination in 10% neutral buffered formalin and for protein studies at −80 °C.

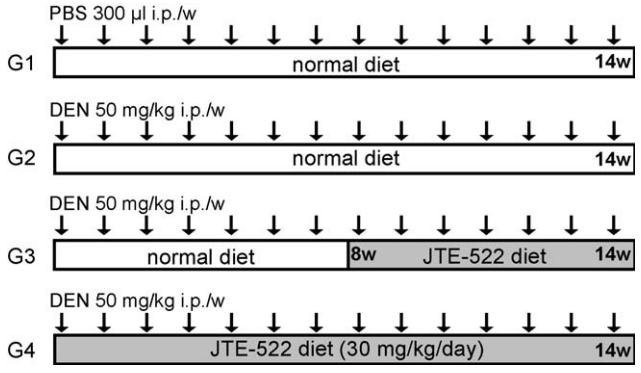

Fig. 1. Experimental schedules. Experimental schedules of the G1 to G4 groups are demonstrated. The animals were randomized, divided into four groups (n = 10 in each group). Animals in group 1 (G1) were intraperitoneally injected with 300 μl of phosphate buffered saline (PBS) weekly for 14 weeks. Animals in groups 2 (G2), groups 3 (G3), and groups 4 (G4) were intraperitoneally injected with DEN (50 mg/kg body weight) in PBS weekly for 14 weeks. In G3 and 4, the rats were fed with JTE-522 in powder form (30 mg/kg) by daily gavage for 7 and 14 weeks, respectively.

2.3. Measurement of serum transaminase and total bilirubin

Serum transaminase (AST and ALT) and total bilirubin levels were measured at SRL, Inc. (Tokyo, Japan).

2.4. Total protein preparation and Western blotting

The liver samples were mashed with BioMasher (Nippi Inc., Tokyo, Japan) and lysed in radioimmune precipitation (RIPA) buffer (Millipore Corp., Bedford, MA, USA) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor mixture tablet (Roche Diagnostics, Basel, Switzerland) for 10 min on ice. Total protein samples (10 μg) were separated on a sodium lauryl sulfate (SDS)-polyacrylamide gel (PAGE) (SuperSep, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). After the membranes were blocked in 5% non-fat milk (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) for 1 h at room temperature, they were probed with primary antibodies overnight at 4 °C, washed three times in TBST, and incubated with antimouse or antirabbit horseradish peroxidase (HRP) antibody in TBST for 1 h at room temperature. After the signals were developed with a chemiluminescence solution (ECL, GE Healthcare Ltd., Buckinghamshire, UK), they were visualized and quantified by an image analyzer (LAS-3000mini, FUJIFILM Co., Tokyo, Japan).

2.5. Histology and immunohistochemistry

The rat liver tissues were fixed in 10% neutral buffered formalin and paraffin embedded. For histologic analysis, serial sections (5 μm) were stained with hematoxylin and eosin (H&E). Neoplastic nodules and HCC were classified on the basis of the published criteria [13]. For immunohistochemistry with the PCNA and GST-P antibodies, after routine dewaxing with xylene and hydration through a graded ethanol series, the sections were incubated with 1.5% hydrogen peroxide solution for 15 min at room temperature to quench endogenous peroxidase activity. For 8-OHdG staining, after routine dewaxing and hydration, the sections were boiled with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0) for 10 min in a microwave for antigen retrieval. The sections were washed in PBS, blocked with 1.5% serum solution, and incubated with primary antibodies overnight at 4 °C. After rinsing with PBS, the sections were incubated with biotinylated secondary antibody for 30 min room temperature, and horse HRP-conjugated ABC solution (Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 30 min room temperature. The peroxidase activity was developed with DAB solution (Vector Laboratories, Inc., Burlingame, CA, USA). Counterstaining was performed with hematoxylin. The PCNA and 8-OHdG labeling indices were represented as the percentage of positively stained nuclei by counting 1000 cells in field at × 200 magnification. The GST-P-positive area was