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High Sensitivity of LEC Rats with Chronic Hepatitis to Hepatocarcinogenesis: Decreases in Unscheduled and Replicative DNA Synthesis of the Hepatocytes

Hirofumi Sakamoto,[1] Norimasa Sawada, Yasuhiro Kamimura,[2] Katsuhiko Enomoto and Michio Mori[3]

Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060

We carried out the following three experiments to clarify the mechanism of hepatocarcinogenesis in Long-Evans Cinnamon (LEC) rats. (1) Sensitivity to diethylnitrosamine (DEN): LEC rats (8 and 25 weeks old) without and with hepatitis and age-matched F344 rats were administered an intraperitoneal injection of a low dose of DEN. Eight weeks after the injection, the numbers of glutathione-S-transferase placental-form (GST-P)-positive foci in the 33-week-old LEC rat liver were significantly higher than those in the livers of the other three groups of rats. (2) Potential for unscheduled DNA synthesis (UDS): Isolated hepatocytes of 25-week-old LEC rats with chronic hepatitis showed about one-third the level of UDS induced by UV irradiation, as compared to that of age-matched F344 rats, while no significant difference was found between the UDS of isolated hepatocytes of 8-week-old LEC rats and age-matched F344 rats. (3) Potential for proliferation: Isolated hepatocytes from 8-week-old LEC rats responded well to epidermal growth factor (EGF) in culture, to almost the same degree as F344 rat hepatocytes, while a remarkable decrease in the responsiveness of hepatocytes isolated from 25-week-old LEC rats to EGF was found. These results suggested that LEC rat hepatocellular carcinoma could be naturally initiated after the onset of hepatitis by carcinogens contaminating food and the environment, probably due to the reduction of DNA repair activity, after which initiated hepatocytes selectively proliferate in response to growth stimuli endogenously produced as a result of continuous loss of hepatocytes (chronic hepatitis), because of a decrease in growth activity of non-initiated hepatocytes.

Key words: LEC rat — Chronic hepatitis — Hepatocarcinogenesis — UDS — Replicative DNA synthesis

The LEC[4] rat is a new mutant inbred strain, which suddenly manifests liver cell injury (hepatitis) with severe jaundice and anemia 16–22 weeks after birth,[1] for which a single autosomal recessive gene is responsible.[2] About 40% of the animals die of hepatic failure by submassive necrosis of hepatocytes. The surviving rats suffer from continuous hepatitis and spontaneously develop enzyme-altered foci in the livers.[3] The number and size of these foci increase with age. Eventually, there is a high incidence of HCC in long-surviving LEC rats about 1 year after birth. Recently, abnormal copper[4] and iron[5] accumulations were demonstrated in the LEC rat liver and the manifestation of hepatitis was attributed to the former.[6] On the other hand, the mechanisms of the development of HCC in the LEC rat liver have not been clarified. In this study, we performed the following three experiments to elucidate the mechanisms of hepatocarcinogenesis in LEC rats. (1) The sensitivity of LEC rat hepatocytes to carcinogens was examined by counting the number of GST-P-positive foci after a single administration of a low dose of DEN. (2) DNA repairing ability of LEC rat hepatocytes was examined by measurement of UDS after UV irradiation in primary culture. Since a decrease in the activities of first-phase drug-metabolizing enzymes was reported in LEC rat liver,[7] we used UV irradiation, which directly damages DNA. (3) Growth activity of LEC rat hepatocytes was investigated by determination of replicative DNA synthesis in primary culture using EGF, which is an effective growth stimulant for hepatocytes.

MATERIALS AND METHODS

Animals Male LEC rats and F344 rats (Charles River Japan, Inc., Atsugi) were employed at the 8th week and the 25th week after birth. Rats were maintained under

[1] Present address: Department of Internal Medicine (Section 1), Sapporo Medical University School of Medicine.

[2] Present address: Toxicology Laboratory, Yoshitomi Pharmaceutical Industries, Ltd., Fukuoka 871.

[3] To whom correspondence should be addressed.

[4] Abbreviations: LEC, Long-Evans Cinnamon, DEN, diethylnitrosamine; GST-P, glutathione-S-transferase placental-form; UDS, unscheduled DNA synthesis; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; F344, Fischer 344; BrdU, bromodeoxyuridine; DAB, 3,3′-diaminobenzidine tetrahydrochloride; TCA, trichloroacetic acid; L-15, Leivobitz's L-15 tissue medium; DMEM, Dulbecco's modified Eagle's medium with high glucose; F-12, Ham's F-12 nutrient mixture; DMN, dimethylnitrosamine.

conventional conditions with temperature and light controls, and fed a basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and water *ad libitum*. LEC rats were divided into two groups, i.e., rats without liver injury (8 weeks after birth) and rats with liver injury (25 weeks after birth). Age-matched male F344 rats were used as counterparts.

Administration of DEN Fig. 1 shows the regimen for detecting initiated hepatocytes. Briefly, rats were administered an intraperitoneal injection of 2, 10 or 50 mg DEN/kg body weight and were killed at the 8th week after the DEN injection. The liver tissues were fixed with ice-cold acetone, embedded in paraffin and sectioned for hematoxylin and eosin staining and for immunohistochemical examination of GST-P. Immunohistochemistry of GST-P was carried out by using the avidin-biotin peroxidase complex method as previously described.[8] The GST-P-positive foci larger than 0.01 mm^2 were counted. The numbers per square centimeter and the size (mm^2) were measured by a Personal Image Analysis System LA-555 (PIAS, Osaka).

Isolation of rat hepatocytes Hepatocytes were isolated by the collagenase perfusion technique.[9] The viability of the isolated cells after isodensity Percoll centrifugation[10] was greater than 95% as determined by the trypan blue exclusion method. The cells in L-15 supplemented with 0.5 μg/ml insulin and 0.1% bovine serum albumin were plated on 60-mm collagen-coated plastic culture dishes at a cell density of 2×10^6 cells/dish for measurement of UDS. For measurement of replicative DNA synthesis, 2×10^5 cells were plated on 35-mm plastic culture dishes coated with collagen type I. After a 2-h attachment period, cells were rinsed with the medium and further cultured in the appropriate medium for each experiment.

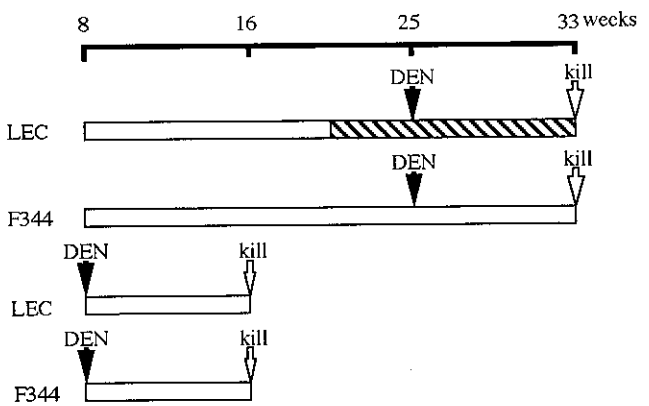

Fig. 1. The regimen for detecting initiated hepatocytes. The slashed bars show the period of manifestation of hepatitis in LEC rats.

Measurement of UDS Measurement of UDS was performed by the method of Plesko and Richardson[11] with some modification.[12] Cells were cultured for 2 h and irradiated with a 15-W germicidal lamp in the presence of 1 m*M* hydroxyurea and 5 μCi of [methyl-^{3}H]thymidine. After 2-h incubation, the cells were harvested with a rubber policeman, then centrifuged and resuspended in Ca^{2+}-free Hanks' balanced salt solution with 2% Triton X-100 for cell lysis. The lysate was centrifuged to pellet the nuclei. Thereafter, DNA, RNA and nucleoprotein were precipitated by the addition of 10% TCA. The precipitate was suspended in 0.5 *N* NaOH and incubated at 37°C for 1 h to hydrolyze RNA. To hydrolyze DNA, the pellet was boiled in 10% TCA for 15 min. After centrifugation, the radioactivity in the supernatant was counted with a scintillation counter (Beckman 5801).

Determination of responsiveness to EGF DNA synthesis was initiated by the addition of 5, 10 or 20 ng of EGF (Sigma Chemical Company, USA)/ml in DMEM/F12 mixtures supplemented with insulin, transferrin, selenium and dexamethasone.[13] After a 30-h incubation, 20 μ*M* BrdU (Amersham, USA) was added to the medium for detection of replicative DNA synthesis. Twenty-four hours later, cells were fixed with 99% ethanol and reacted with anti-BrdU antibody (Dakopatts a/s, Denmark), followed by staining with DAB (Katayama Chemical Co.).[14] The BrdU labeling index was determined.

RESULTS

In 16-week-old LEC rats and age-matched F344 rats, administered single intraperitoneal injections of low doses of DEN at the 8th week after birth, virtually no GST-P-positive foci were found in the livers (Table I). The numbers of GST-P-positive foci in the livers of 33-week-old LEC rats, treated with DEN at the 25th week after birth, were significantly higher than those of spontaneous GST-P-positive foci in 33-week-old LEC rats without treatment. The initiation effect of DEN was shown to be dose-dependent in livers of 25-week-old LEC rats. In the livers of 33-week-old F344 rats, however, few spontaneous GST-P-positive foci were found, and the numbers of GST-P-positive foci in F344 rat livers did not increase with the dose of DEN, showing that the doses of DEN used in the present study were too low to induce GST-P-positive foci in the F344 rat liver. The average size of GST-P-positive foci initiated with DEN was smaller than that of spontaneous GST-P-positive foci in 33-week-old LEC rat livers but the difference was not statistically significant.

In the UDS experiment, the level of UDS of 25-week-old LEC rat hepatocytes was significantly lower than that of age-matched F344 rats by about one-third and

than that of 8-week-old LEC rat hepatocytes by about one-half (Fig. 2). In F344 rats, the level of UDS of 25-week-old rats was significantly higher than that of 8-week-old F344 rats ($P<0.001$), which is consistent with the results reported previously.[11] On the other hand, hepatocytes isolated from 8-week-old LEC rats showed the same level of UDS as observed in 8-week-old F344 rat hepatocytes. Thus, the reduced level of UDS in LEC rat hepatocytes became clear after the beginning of continuous hepatocyte renewal. The measurement of UDS is affected by the thymidine pool in the cell. Though the pool was not determined, the level of UDS was measured at a concentration (0.2 μM) of thymidine sufficient to saturate the thymidine pool.[15] As was expected, the level of UDS was reduced in each group. However, the significant differences between groups remained (data not shown).

The responsiveness of hepatocytes isolated from 8-week-old LEC rats to EGF was almost the same as that of hepatocytes isolated from age-matched F344 rats, while a significant decrease in the responsiveness of 25-week-old LEC rat hepatocytes to EGF was found

Table I. Mean Numbers of GST-P-positive Foci in the Livers of LEC Rats and F344 Rats, Treated with a Single Administration of DEN

Animal	Dose of DEN (mg/kg body wt.)	No. of animals	No. of foci (/cm²)	Average size of foci (mm²)
LEC 16W	0	4	0.07±0.12	0.02
	2	3	0	
	10	7	0	
	50	4	0	
LEC 33W	0	9	1.06±0.65[a)]	0.14±0.10
	2	7	3.34±0.91[a, b)]	0.08±0.04
	10	8	7.13±2.18[b, c)]	0.07±0.03
	50	7	11.41±4.20[c)]	0.06±0.02
F344 16W	0	3	0	
	2	3	0	
	10	3	0	
	50	4	0	
F344 33W	0	3	0.90±0.17	0.02±0.01
	2	3	0.39±0.29	0.03±0.00
	10	3	0.09±0.12	0.01±0.23
	50	4	0.58±0.33	0.06±0.02

a and *b*) $P>0.01$. *c*) $P>0.05$.

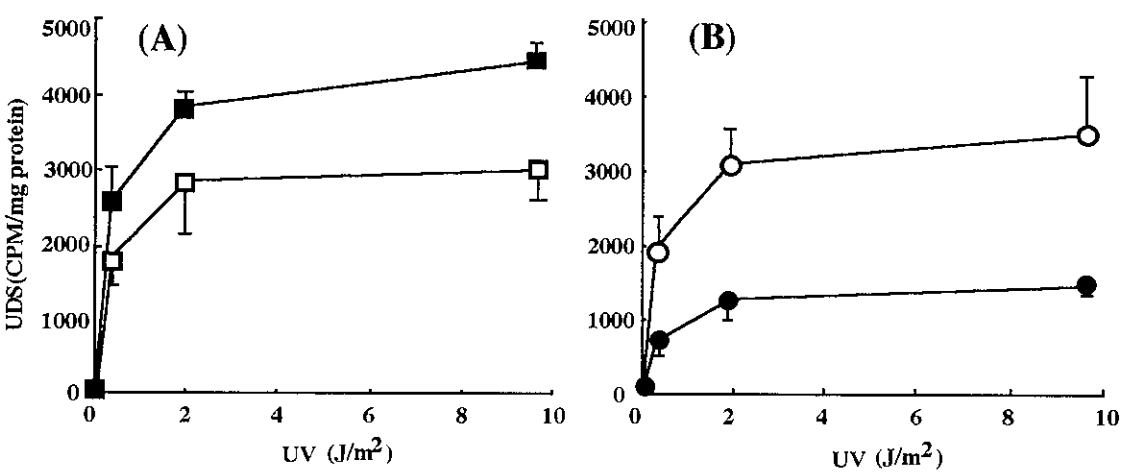

Fig. 2. UDS induced by UV irradiation of primary cultures of hepatocytes isolated from F344 rats (A) and LEC rats (B). Open squares and circles, 8-week-old rats; closed squares and circles, 25-week-old rats.

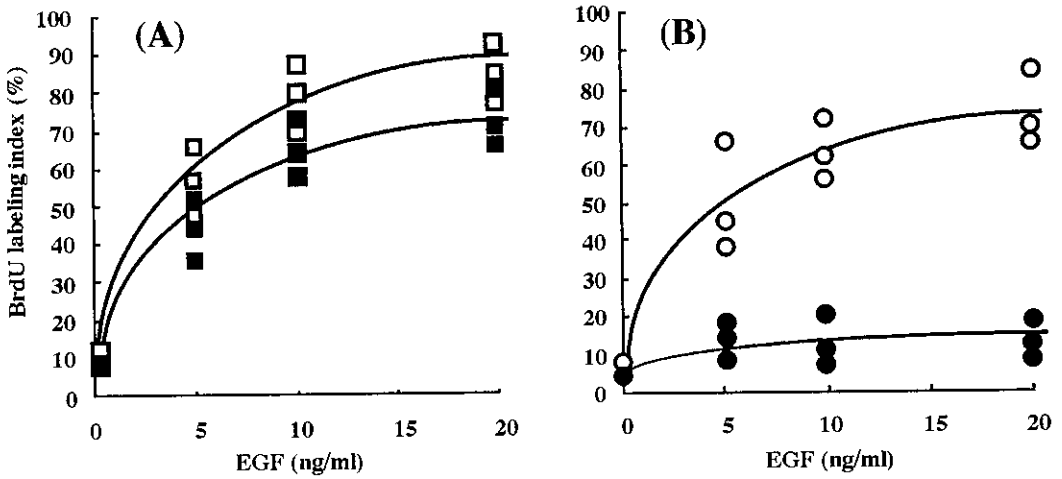

Fig. 3. Responsiveness of hepatocytes to EGF isolated from F344 rats (A) and LEC rats (B). Open squares and circles, 8-week-old rats; closed squares and circles, 25-week-old rats.

(Fig. 3). These results clearly showed that the majority of hepatocytes of LEC rats with chronic hepatitis had lost growth activity. On the other hand, mitosis of hepatocytes in the liver is frequently observed in hepatitis, suggesting that a limited cell population including both non-initiated and initiated hepatocytes actively proliferates in the case of continuous loss of hepatocytes in the liver.

DISCUSSION

In this study, large numbers of GST-P-positive foci were induced in a dose-dependent manner by a single administration of a low dose of DEN in the liver in LEC rats with chronic hepatitis, while no focus was induced by DEN at low doses in age-matched F344 rat livers and LEC rat livers before the onset of hepatitis. These results indicate that the livers of LEC rats with chronic hepatitis were much more sensitive to the initiating effect of DEN.

Recent advances in molecular biotechnology have led to the development of several interesting transgenic mice[16-22] which can be used to study the mechanisms of hepatocarcinogenesis. Using these mice, several groups[20-22] stressed that an active proliferation of hepatocytes, resulting from degenerative cell death and/or apoptosis, occurs in the liver prior to the development of cancer. In the LEC rat, we demonstrated that the rats with chronic hepatitis were far more sensitive to DEN than the rats without hepatitis, though the rats without hepatitis were still sensitive to carcinogens as compared to the other rat strains.[23] Furthermore, the hepatitis-HCC sequence is frequently observed in human hepatocarcinogenesis. Thus, active renewal of hepatocytes seems to be one of the most important phenomena, regardless of cause, for the development of HCC, as reviewed previously.[24]

In the present experiments, we examined the potentials for UDS[25] and for growth[24] of LEC rat hepatocytes, as possible mechanisms of the high susceptibility of LEC rats to the initiation of hepatocarcinoma. The levels of UDS induced by UV-irradiation were measured in LEC rat hepatocytes, revealing that the level of UDS was significantly reduced in hepatocytes isolated from LEC rats with chronic hepatitis. This observation suggests that the high sensitivity to DEN, in terms of development of GST-P-positive foci, of LEC rat livers after the onset of hepatitis may be attributable to the reduction of UDS. Furthermore, since the majority of hepatocytes from LEC rats with chronic hepatitis lose growth activity, a limited cell population in the liver may go through several cell cycles in response to the growth stimuli endogenously induced by continuous loss of hepatocytes. Thus, the multiple repetition of cell division may also result in high susceptibility to the initiating effects of carcinogens.

Taking all the results together, we consider that the LEC rat is highly susceptible to carcinogens, probably due to decreased levels of UDS after suffering from hepatitis and/or due to frequent cell division of a limited cell population. Therefore, initiated hepatocytes could selectively proliferate in LEC rat liver due to reduced proliferation activity of the surrounding normal hepatocytes in response to continuous loss of hepatocytes. Thus,

the LEC rat is not only useful for screening unknown hepatocarcinogenic agents or weak carcinogens but also is an interesting animal for analyzing the multistep process of hepatocarcinogenesis.

Recently, there have been reports that copper accumulates in LEC rat liver[4] and that penicillamine, a chelating agent for copper, protects against the manifestation of hepatitis,[26] and a genetic linkage between copper accumulation and the manifestation of hepatitis was proved by measurement of the copper concentrations in the livers of backcrosses of LEC F_1.[6] These reports suggest that accumulation of copper in the LEC rat hepatocyte can cause liver cell injury. However, the relationship between hepatocarcinogenesis and accumulation of copper is unclear. Direct attack of copper on DNA was reported *in vitro*,[27] but the incidence of HCC in rats given DMN and cupric acetate was only about 40% of that in rats given DMN alone.[28] Recently, in addition to copper, the accumulation of iron was reported in LEC rat livers.[5] Copper and iron are known to produce free radicals, which may induce hepatocyte injuries, involving damage to DNA.[29] In LEC rat livers, the contents of copper and iron were reported to be almost constant after the 3rd month[4] and 13th week[5] of age respectively, suggesting that the accumulations of copper and iron in the liver do not directly influence growth and DNA repair activity.

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