ORIGINAL

Low susceptibility to *N*-ethyl-*N*-nitrosourea-induced transplacental carcinogenesis in Long-Evans Cinnamon (LEC) rats

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Abstract : The Long-Evans Cinnamon (LEC) rat, an animal model of Wilson's disease, is resistant to a variety of chemical carcinogenesis except liver and colon. In the present study, *N*-ethyl-*N*-nitrosourea (ENU)-induced transplacental carcinogenesis was examined in male and female LEC, Long-Evans Agouti (LEA), a sibling line of the LEC rat, and F344 rats (n=21). ENU was administered to pregnant rats as a single s.c. injection at a dose of 60 mg/kg body weight on the 17th day after conception. Cerebral/spinal gliomas and trigeminal/spinal nerve schwannomas developed in both LEA and F344 rats at 30 weeks of age, but no nervous system tumors developed in LEC rats, the difference being statistically significant. Lung adenomas also developed in LEA and F344 rats, but not in LEC rats. Semiquantitative RT-PCR demonstrated that metallothionein (MT)1a, MT2 and O^6-methylguanine-DNA methyltransferase (MGMT) mRNA levels in the liver of LEC rats were higher than those in F344 and LEA rats. In addition, Western blot analysis showed that MT (MT1 plus MT2) in the liver of LEC rats was also higher than that in other strains. Present results suggest that high levels of MT and/or MGMT contribute to the resistance to nitrosamine-induced carcinogenesis in LEC rats. J. Med. Invest. 56 : 93-98, August, 2009

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INTRODUCTION

The Long-Evans Cinnamon (LEC) rat has abnormalities in copper excretion in the liver and T-cell maturation in the thymus (1, 2). Excess copper induces toxic hepatitis while liver and kidney tumors develop spontaneously in LEC rats (3, 4). A recent our report indicates that inflammatory bowel disease- like colitis develops in the LEC strain as a consequence of regulatory T-cell dysfunction making this strain is susceptible to *N*-methyl-*N*-nitrosourea (MNU) and azoxymethane (AOM)-induced colon carcinogenesis (5). In contrast, our previous studies demonstrated that LEC rats are resistant to *N*-diethylnitrosamine (DEN), MNU and to *N*-nitrosobis (2-hydroxypropyl)amine(BHP)-induced lung carcinogenesis, and *N*-butyl-*N*-(hydroxybutyl) nitrosamine(BBN)-induced bladder carcinogenesis in

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our studies (6, 7).

Prenatal and neonatal administrations of *N*-ethyl-*N*-nitrosourea (ENU) induce brain and trigeminal nerve tumors (8). Intracranial schwannomas in rats originate in trigeminal nerve, but in humans, trigeminal nerve is the second most frequent site next to the vestibular nerve (9). ENU is an alkylating agent and it was demonstrated that the exposure to ENU causes O^6-alkylguanine formation in DNA (10), and the adduct is removed by repair enzymes such as O^6-methylguanine-DNA methyltransferase (MGMT) (11, 12).

In the present study, we investigated the ENU-induced transplacental carcinogenesis and the mechanisms of cancer susceptibilities in the three strains of rats.

MATERIALS AND METHODS

Animals

Inbred LEC/Tj and LEA/Tj rats were bred in the Institute for Animal Experimentation of the University of Tokushima, Tokushima, in specific pathogen-free conditions. F344/DuCrj rats were purchased from Charles River Japan, Inc., Kanagawa, Japan. Animals were housed three in a plastic cage with sterilized woodchips for bedding in an air-conditioned room at 23± 2°C and 55± 10% humidity with a 12 h light/dark cycle. They were fed a pellet diet (Oriental Yeast Co., Tokyo, Japan) and received tap water *ad libitum*. Experiments were conducted according to the Guideline for the Care and Use of Laboratory Animals of the University of Tokushima Graduate School, and all experimental protocols were approved by the Animal Committee.

Carcinogenicity study

Male and female rats (n=21) of LEC, LEA and F344 strains were used. Pregnant rats were given s.c. injections of 60 mg/kg ENU (Sigma Chemical Co., St. Louis, MO.) dissolved in 0.1 M citrate buffer (pH 6.0) on the 17th day after conception. Vaginal smears were examined every morning to confirm the conception. Body weight was monitored every other week, and all surviving animals were killed at 30 weeks of age under carbon oxide narcosis. The brain, spinal cord, lungs, kidneys, soft tissues and bone were fixed in 10% buffered formalin, embedded in paraffin. For histological examination, sections were stained with hematoxylin and eosin.

Semiquantitative RT-PCR

Livers from male F344, LEA, and LEC rats (9 weeks old for MT1a and MT2 mRNA, 21 weeks old for MGMT mRNA) were stabilized with RNAlater (Ambion) for over night at 4°C and then total RNAs were isolated using an RNeasy® Mini Kit (QIAGEN). By using 2 μg (for MT1a and MT2 mRNA) or 5 μg (for MGMT mRNA) aliquots, cDNAs were synthesized with SuperScript II reverse transcriptase and random hexamers (Invitrogen). Reactions were conducted at 42°C for 60 min, after the increase of temperature to 72°C for 15 min. The total cDNAs were then amplified by PCR following a thermocycling program at 94°C for 10 min for initial denaturation, 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min for amplification, and a final extension at 72°C for 10 min for MT1a, MT2, and β-actin or 95°C for 5 min for initial denaturation, 95°C for 30 s, 50°C for 3 min, and 72°C for 3 min for amplification, and a final extension at 72°C for 10 min for MGMT, respectively. The numbers of amplification cycles were as follows : MT1a and MT2, 25 cycles ; MGMT, 45 cycles ; and β-actin, 23 cycles. PCR reactions were visualized by ethidium bromide after separating by 1.5% agarose gel electrophoresis. Sequences of primers were : rMt1a-F 5'-AACTGCTCCTGCTCC-ACC-3', rMt1a-R 5'-TCAGGCACAGCACGTGCA-3', rMt2-F 5'-AACTGCTCCTGTGCCACAG-3', rMt2-R 5'-CTCTTTGCAGATGCAGCC-3', rMGMT3-F 5'-AGCCTGGCTGGAAGCCTA-3', rMGMT3-R 5' AT-TGCTCCTCATCGCTCC 3', β-actin-F 5'-TACAAT-GAGCTGCGTGTGG-3', and β-actin-R 5'-AGATG-GGCACAGTGTGGG-3'.

Western blot analysis

Livers from male F344, LEA, and LEC rats (9-weeks old) were lysed with radioimmunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] and the protein concentrations of the samples were quantified using a DC Protein Assay Kit (Bio-Rad). Next, aliquots (25 μg protein) were subjected to SDS-PAGE (12% gel) and transferred to polyvinylidene difluoride membranes. Mouse monoclonal antibody against metallothionein (Clone UC1MT, QED Bioscience) and rabbit polyclonal antibody against actin (Sigma) were used as the primary antibodies. Anti-metallothionein recognizes both MT1 and MT2. Goat anti-mouse IgG-horseradish peroxidase (American Qualex) and Goat

anti-rabbit IgG-horseradish peroxidase (Invitrogen) were employed as the secondary antibodies. The dilution rates were as follows : anti-metallothionein (1 : 1,000), anti-actin (1 : 8,000), anti-mouse IgG-horseradish peroxidase (1 : 50,000), and anti-rabbit IgG-horseradish peroxidase (1 : 150,000). An Immobilon Western horseradish peroxidase substrate (Millipore) was applied to detect the signals.

Statistical analyses

The incidences of tumors were analyzed by Fisher's exact probability test.

RESULTS

ENU-induced carcinogenesis

Six of male and ten of female LEC rats died of copper-mediated hepatic injury by 21 weeks old and 17 weeks old, respectively. Table 1 shows the incidences of tumors in three strains of rats. The central and peripheral nervous system and the lung were the major target organs of ENU in LEA and F344 rats. There were no significant sex differences in all neoplasms. In contrast, no nervous system and lung tumors developed in male and female LEC rats, the difference being significant from LEA and F344 rats. Soft tissue and bone tumors developed in LEA and F344 rats at low incidences, but no tumors were observed in LEC rats. Histologically, brain tumors were gliomas, and peripheral nervous system tumors were malignant schwannomas (Fig. 1). All lung tumors were adenomas.

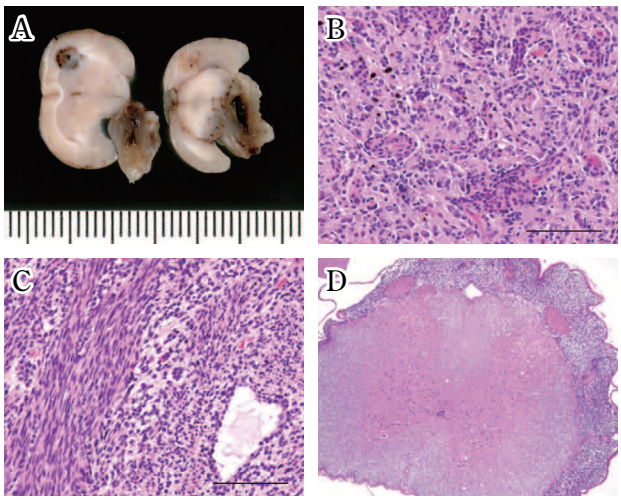

Fig. 1. Gross and histological appearance of the tumors. A, Cerebral and trigeminal nerve tumors (F344, male). B, Cerebral glioma (F344, male). C, Trigeminal schwannoma (LEA, male). D, Malignant schwannoma of the spinal nerve (F344, female). Bar, 100 μm.

Expression of MT and MGMT

In LEC strain, MT1a and MT2 mRNA levels in the liver were clearly higher than those in other strains (Fig. 2A). Western blot analysis revealed that protein level of MT (MT1 plus MT2) in LEC strain was extremely higher than that in other strains (Fig. 2B). Like the result of MT, MGMT mRNA level in LEC strain was clearly higher than that in other two strains (Fig. 2C). We tried Western blotting of MGMT using anti-mouse antibody, but the suitable

Table 1. Incidences of *N*-ethyl-*N*-nitrosourea-induced tumors in male and female LEC, F344 and LEA rats.

	Strain	Effective no. of rats	Tumors				
			Brain and spinal cord	Trigeminal and spinal nerve	Lung	Kidney	Soft tissue and bone
Male							
	LEC	15[a]	0[c,e]	0[d,e]	0[c]	0	0
	LEA	21	9(43%)	14(67%)	12(57%)	0	4(19%)
	F344	21	7(33%)	8(38%)	5(24%)	0	1(5%)
Female							
	LEC	11[b]	0[e]	0[c]	0	1(9%)	0
	LEA	21	4(19%)	11(52%)	4(19%)	2(10%)	3(14%)
	F344	21	8(38%)	6(29%)	0	0	1(5%)

[a]Six rats died of hepatic injury between 17 and 21 weeks old.
[b]Ten rats died of hepatic injury between 15 and 17 weeks old.
[c,d]Significantly different from LEA rat at [c]$P < 0.01$, [d]$P < 0.001$ by Fischer's exact probability test.
[e]Significantly different from F344 rat at $P < 0.05$ by Fischer's exact probability test.

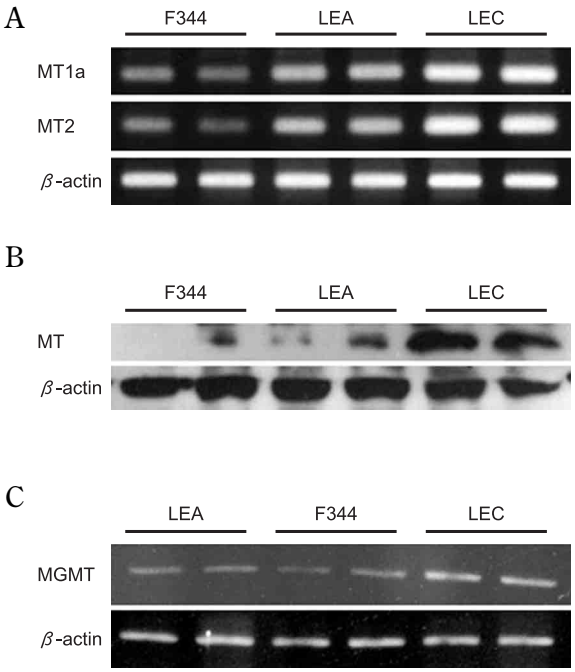

Fig. 2. Expression of MT1a and MT2 mRNA, MT protein, and MGMT mRNA in the liver of three strains. MT1a and MT2 mRNA (A), MT protein (B), and MGMT mRNA (C) were detected by semiquantitative RT-PCR and Western blot analysis. In both experiments, β-actin was also detected as an internal standard. The sizes of amplified PCR products for MT1a, MT2, MGMT, and β-actin are 177, 147, 223, and 226 bp, respectively.

antibodies that react to rat MGMT protein were not obtained at present. Although data was not shown, we examined the expression level of MGMT mRNA in the brain, but no expression was observed in all strains.

DISCUSSION

We first described that the LEC rat is an entirely resistant strain to ENU-induced transplacental carcinogenesis of the nervous system and lung. Strain difference and genetic susceptibilities to ENU-induced neural tumors in rats have been investigated (13-15). Indeed, it is known that BDIV is a resistant strain while BDIX and BDVI are susceptible strains (13). Wistar-Furth rats are more resistant than Long-Evans rats and males are more susceptible than females (14), whereas our data showed no sex differences in the incidences of neuronal tumors. An analbuminemic rat is a susceptible strain than control Sprague-Dawley rats, although O^6-ethylguanine formation and elimination in the fetal brain are not different in both strains (15). Recently, multiple tumor susceptibility/resistance loci of ENU-induced malignant schwannomas in BD strains are mapped on chromosome 10 *et al.* (16, 17).

It has been demonstrated that MGMT exerts a protective effect on nitrosamine-induced liver and lung carcinogenesis in the studies using knockout and transgenic mouse models (18-20). Metallothionein (MT) is a cysteine rich metal-binding protein that is inducible by endogenous and exogenous stimuli and has protective roles in apoptosis and drug resistance (21). It has been pointed out that MT exerts a protective effect on skin, kidney and bladder carcinogenesis in the studies using MT-I/II deficient mice (22-24). We showed that MGMT and MT in the LEC rat liver were higher than those in LEA and F344 strains by semiquantitative RT-PCR and Western blotting. Data on knockout/transgenic models suggest that the overexpression of both MGMT and MT exerts an important role in the low susceptibility of LEC rats to ENU-induced transplacental carcinogenesis.

Oligonucleotide microarray analyses using Rat oligomicroarray kit (Agilent Technologies) were performed. Accordingly, gene expression of MT and MGMT in the liver of 21-week-old male LEC rats was >96.5 and >3.5 times those in LEA and F344 rats, respectively. These data are consistent with a previous report (25).

Taken together, our data suggest that the overexpression of both MT and MGMT would exert an additive protective effect on ENU-induced carcinogenesis in LEC rats. Further experiments are necessary to elucidate the role of these and other genes participating cancer susceptibility in LEC rats.

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