High Susceptibility to Zymbal Gland and Intestinal Carcinogenesis in Diabetic Otsuka Long-Evans Tokushima Fatty Rats

Ezar Hafez1, Tetsuyuki Takahashi1, Hirohisa Ogawa1, Makoto Sato1, Tokiko Nakai1, Chie Takasu1, Hisanori Uehara1, and Keisuke Izumi1

1Department of Molecular and Environmental Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

Abstract: Diabetes mellitus (DM) and obesity are believed to be risk factors for colorectal cancer in humans. In experiment 1, male nondiabetic Long-Evans Tokushima Otsuka (LETO) rats and Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model animal of type 2 DM, were whole-body X-irradiated (4 Gy) at 6 and 8 weeks of age and euthanized at 78 weeks of age (n=15, respectively). The incidences of small intestine adenocarcinoma in LETO and OLETF rats were 0% and 30%, respectively. In experiment 2, male LETO and OLETF rats (n=24, respectively) were given s.c. injections of 15 mg/kg azoxymethane (AOM) once weekly for 3 weeks and euthanized at 36 weeks of age. The incidences of Zymbal gland tumors in LETO and OLETF rats were 0% and 67%, respectively (P<0.001), whereas those of small intestine adenocarcinoma were 0% and 67%, respectively (P<0.001), and those of cecum/colon adenocarcinoma were 46% and 79% (P<0.05), respectively. Fatty change of hepatocytes was common in OLETF rats (63%) but not in LETO rats. Serum triglyceride and free fatty acid levels in OLETF rats were significantly higher than in LETO rats at sacrifice, whereas serum insulin levels in OLETF rats were very diverse. These data suggest that hyperlipidemia plays a significant role in high susceptibility to lower intestinal tract carcinogenesis in OLETF rats; this strain is susceptible to AOM-induced Zymbal gland carcinogenesis. (DOI: 10.1293/tox.24.187; J Toxicol Pathol 2011; 24: 187–193)

Key words: OLETF rat, carcinogenesis, zymbal gland, colon, hyperlipidemia

Introduction

Epidemiologic studies have suggested a positive link between diabetes mellitus (DM) and various neoplasms including colon cancer1-2. Obesity is a risk factor for DM and cancer of various organs including the colon3-4. Insulin resistance, chronic inflammation and/or altered adipokine secretion with resultant hyperlipidemia may be involved in DM and obesity-related cancer5. Exogenous insulin use may also be a risk factor for colorectal cancer6.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats7, established from a closed colony of Long-Evans rats by selective breeding along with nondiabetic Long-Evans Tokushima Otsuka (LETO) rats, are a model animal of type 2 DM and show late conversion to insulin-dependent disease. This strain has mild obesity, and a homozygous deletion in cholecystokinin-1 (CCK1) receptor gene responsible for hyperphagia has been identified8,9. OLETF rats are susceptible to N-nitrosobis(2-oxopropyl)amine-induced thyroid carcinogenesis10. In the present study, we examined X-irradiation and azoxymethane (AOM)-induced carcinogenesis in LETO and OLETF rats.

Materials and Methods

Animals

LETO and OLETF rats were obtained from Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan. Animals were housed three to 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 and given pellet diet (Oriental MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. Experiments were conducted according to the Guidelines for the Care and Use of Laborato-
Pure Chemical Industries, Ltd., Osaka, Japan) was given by 16-h fasting in experiment 1 (n=9). Glucose (Wako rats were submitted to an oral glucose tolerance test (OGTT) examined histologically. At 12 and 42 weeks of age, control with hematoxylin and eosin. Other major organs were also
fixed in 10% buffered formalin and embedded in paraffin. For histological examination, tissue sections were stained
and location of tumors were recorded; the tumors were then
thesized at 78 weeks of age under carbon dioxide narco-
was monitored every other week, and all animals were eu-
for 3 weeks (Fig. 1). AOM was dissolved in 0.9% NaCl just
mg/kg body weight, at about 10 a.m. Blood was col-
lected from the tail. Thirty-minute postglucose load blood
glucose was measured by the glucose oxidase method. Se-
rum insulin was measured using an ELISA kit (Morinaga
insulae-like growth factor (IGF)-1, plasminogen activator in-
hibitor (Pai)-1 and β-actin, according to the manufacturer’s
guidelines. β-Actin was used as an internal standard. These
samples were then amplified using a 7500 Real-time PCR
System (Applied Biosystems), and the data were analyzed
with 7500 System SDS software (Applied Biosystems). Thermocycling program was as follows: 95 °C for 30 s for
initial denaturation and 40 cycles of 95 °C for 5 s and 60 °C
for 34 s for amplification. The sequences of the primers
were as follows: IGF-1-F, 5′-TTCAGTTCGTGTTGTGGAC-
CAAG-3′; IGF-1-R, 5′-GATCACAGCTCCGGAAGCAA-3′;
Pai-1-R, 5′-GGAGATTACTGCCCTGGCTCCTA-3′; and β-actin-R,
5′-GACTCATCGTACTCCTGCTTGCTG-3′.

Measurement of food intake
Male LETO and OLETF rats were given pelleted diet, and
food intake and body weight were monitored every other
week until 20 weeks of age (n=15, respectively).
Statistical analyses
The incidence of tumors was analyzed by Fisher’s ex-
act probability test; other data were analyzed by two-tailed
Student’s t-test.

Results

Experiment 1
The Average body weight of X-irradiated OLETF rats was found to be increased at sacrifice because of huge
soft tissue sarcomas in some rats (Fig. 2). X-irradiation increased the incidence of various tumors in both strains
(Table 1). Small intestinal adenocarcinomas developed in 3 OLETF rats (30%), but not in LETO rats. The colonic tu-
mor in the X-irradiated OLETF rat was a leiomysarcoma. X-irradiation caused a low incidence of islet cell tumors
in both strains. Hyperglycemia at 12 and 42 weeks of age and hyperinsulinemia at 42 weeks of age were observed in
control OLETF rats (Fig. 3). X-irradiation increased blood glucose levels and reduced insulin levels in OLETF rats
compared with the control at 42 weeks of age, although not
significantly, whereas these parameters were not affected by
X-irradiation in LETO rats.

**Experiment 2**

The average body weight of OLETF rats was higher than that of LETO rats throughout the experimental period (Fig. 4). The incidence of Zymbal gland, small intestine and colon tumors in OLETF rats was significantly higher than in LETO rats (Table 2). Malignant lymphoma developed in one LETO rat, but no other tumors developed in either strain.

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**Table 1. Incidence of Tumors in 4-Gy X-irradiated Male LETO and OLETF Rats**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of rats</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-irradiated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>OLETF</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>4-Gy X-irradiated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>OLETF</td>
<td>10</td>
<td>2 (20%)</td>
</tr>
</tbody>
</table>

* Two rats died of unknown cause at 24 and 45 weeks of age. *b Four rats died of severe diabetes at 12, 12, 13 and 20 weeks of age, and one rat died accidentally at 42 weeks of age.

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**Fig. 2.** Growth curves of male LETO and OLETF rats in X-irradiation-induced carcinogenesis.

**Fig. 3.** Average blood glucose and serum insulin levels in LETO and OLETF rats at 12 and 42 weeks of age in an oral 2 g/kg OGTT (experiment 1). A, sham-irradiated rats; B, X-irradiated (4 Gy) rats. *, ** and ***; P<0.05, P<0.01 and P<0.001, respectively, versus LETO rat (two-tailed Student’s t-test). □, LETO rat; ■, OLETF rat; Bars, SD.

**Fig. 4.** Growth curves of male LETO and OLETF rats in AOM-induced carcinogenesis.

**Fig. 5.** Average no. of small intestine and colon tumors in LETO and OLETF rats (experiment 2). The average no. of colon tumors in OLETF rats was 2.1 times higher than in LETO rats. * and **; P<0.01 and P<0.001, respectively, versus LETO rats (two-tailed Student’s t-test). □, LETO rat; ■, OLETF rat; Bars, SD.

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Hafez, Takahashi, Ogawa et al.
Fig. 6. AOM-induced Zymbal gland tumors in OLETF rats. A, bilateral Zymbal gland tumors in an OLETF rat; B, sebaceous carcinoma of the Zymbal gland. Bar, 100 µm.

Fig. 7. Histological appearance of AOM-treated LETO and OLETF rats (experiment 2). A, liver of a LETO rat; B, liver of an OLETF rat; C, pancreatic islets of a LETO rat; D, pancreatic islets of an OLETF rat. Bars, 100 µm.
The average numbers of small intestine and colon tumors in OLETF rats were higher than in LETO rats (Fig. 5). These tumors were mainly tubular adenocarcinoma histologically, and signet-ring cell carcinoma developed in one LETO rat. In one OLETF rat, colonic cancer metastasized to a retroperitoneal lymph node. Six of the 16 Zymbal gland tumors in OLETF rats, sebaceous carcinoma histologically, were bilateral (Fig. 6). The incidence of fatty change of hepatocytes was 15/24 (63%) in OLETF rats and 0% in LETO rats (Fig. 7). Pancreatic islets of OLETF rats showed hemosiderosis, loss of islet cells and fibrosis. The average serum insulin level in OLETF rats was lower than in LETO rats (Fig. 8). The insulin levels in 2 of 9 OLETF rats were higher than the maximum value in LETO rats, but those in 5 OLETF rats were lower than the minimum value in LETO rats. Serum triglyceride and free fatty acid levels in OLETF rats were significantly higher than in LETO rats at sacrifice.

**Analysis of the liver**

The relative expression levels of liver IGF-1 and Pai-1 mRNA in OLETF rats were 1.08 ± 0.23-fold and 1.63 ± 0.45-fold, respectively, versus LETO rats (Fisher's exact probability test).

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**Table 2. Incidence of AOM-induced Tumors in Male LETO and OLETF Rats**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of rats</th>
<th>Tumors</th>
<th>Zymbal gland</th>
<th>Small intestine</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>LETO</td>
<td>24</td>
<td></td>
<td>0</td>
<td>0</td>
<td>11 (46%)*</td>
</tr>
<tr>
<td>OLETF</td>
<td>24*</td>
<td></td>
<td>16 (67%)**</td>
<td>9 (38%)**</td>
<td>19 (79%)*</td>
</tr>
</tbody>
</table>

* One rat died of colonic and Zymbal gland tumors at 20 weeks of age. Five rats were euthanized at 29, 31, 32, 33 and 34 weeks of age due to colonic and/or Zymbal gland tumors. *, ** and ***; \( P < 0.05 \), \( P < 0.01 \) and \( P < 0.001 \), respectively, versus LETO rats (Fisher's exact probability test).

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**Fig. 8.** Serum insulin, triglyceride, free fatty acid and total cholesterol in LETO and OLETF rats at 36 weeks of age (experiment 2). * and **; \( P < 0.05 \) and \( P < 0.01 \), respectively, versus LETO rats (two-tailed Student’s \( t \)-test). A, insulin; B, triglyceride; C, free fatty acid; D, total cholesterol. □, LETO rat; ■, OLETF rat; Bars, SD.

**Fig. 9.** Expression of IGF-1 and Pai-1 mRNA in the liver of LETO and OLETF rats (\( n=5 \)). Relative expression levels of IGF-1 and Pai-1 mRNA were detected by qRT-PCR. Bars, SD.

**Fig. 10.** Food intake/body weight ratio in LETO and OLETF rats. □, LETO rat; ■, OLETF rat.
fold, respectively, those in LETO rats. No significant differences were observed between the strains (Fig. 9).

**Food intake**

The average food intakes of the untreated LETO and OLETF rats were 18.5 g and 26.0 g, respectively. The food intake/body weight ratio in OLETF rats was 1.08–1.19 times higher than in LETO rats (Fig. 10).

**Discussion**

X-irradiation induced a low incidence of intestinal cancer in OLETF rats, but not in LETO rats, and OLETF rats were clearly susceptible to AOM-induced Zymbal gland and lower intestinal tract carcinogenesis. Zucker obese rats, a model animal with leptin receptor gene mutation with hyperleptinemia, exhibit marked obesity and type 2 DM11,12. Zucker obese rats are also more susceptible to AOM-induced colon cancer than control Zucker lean rats13,14. [REMOVED HYPERLINK FIELD]. The OLETF strain also shows hyperleptinemia, but there is no mutation in the leptin receptor gene15, and obesity is milder than in Zucker obese rats.

Hyperlipidemia is suggested to be an important factor for intestinal polyp formation in Apc-deficient mice from studies on suppression of hyperlipidemia by peroxisome proliferator-activated receptor-α and receptor-γ agonists and by Pasi-1 blockers16,17. In our recent study, 20% caloric restriction reduced serum triglyceride and inhibited both AOM-induced colonic and Zymbal gland neoplasms in OLETF rats (data not shown). Hence, the hyperlipidemic state in OLETF rats may play a significant role in high susceptibility to intestinal carcinogenesis. Insulin and IGF-1, growth factors in vitro, increase in obesity. It is reported that insulin promoted AOM-induced colon carcinogenesis in F344 rats18. In the present experiments, the insulin levels in the OGTT in experiments 1 and 2 were various. Although the measurement conditions were different, AOM treatment might reduce insulin levels.

The Zymbal gland, a sebaceous gland of the external ear canal, is a target organ of AOM in rats19,20. In the present study, Zymbal gland tumor developed at a high rate, 67% in AOM-treated OLETF rats. However, in our recent study performed using the same protocol as the present study in nonobese Long-Evans Agouti rats21, a new model of type 2 DM, the incidences of Zymbal gland, small intestine and colon tumors were 0%, 4% and 83%, respectively (unpublished data). There is no description of Zymbal gland tumor in AOM-treated Zucker obese rats22. Therefore, OLETF rats should be a strain susceptible to AOM-induced Zymbal gland carcinogenesis. It is known that both Zymbal glands and intestines in rats are target organs of heterocyclic amines in cooked foods such as 2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-6-methylidipyrido[1,2-a:3′,2′-d]imidazole and 2-amidopyrido[1,2-a:3′,2′-d]imidazole23. In humans, the familial disease of sebaceous carcinoma plus visceral cancer including colorectal carcinoma is known as Muir-Torre syndrome24, a variant of hereditary nonpolyposis colorectal cancer. The mechanism of susceptibility to Zymbal gland carcinogenesis in OLETF rats should be elucidated.

In conclusion, the hyperphagic OLETF strain is a useful model for investigation of mechanisms of carcinogenesis and cancer prevention in diabetic conditions and obesity, and hyperlipidemia may play an important role in high susceptibility to Zymbal gland and intestinal carcinogenesis in OLETF rats.

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**References**


