Original Article

Female heterozygous (+/fa) Zucker rats as a novel leptin-related mammary carcinogenesis model

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ABSTRACT — The homozygous mutant fatty Zucker rat (*fa/fa*) is the prominent model for the research of obesity, one of the most well-known risk factor of postmenopausal mammary cancer. But the usage as a mammary gland carcinogenesis model is considered to be restricted due to the hypoplasia of mammary gland. In the present study, to find the validity of heterozygous mutant (+/fa) lean Zucker rats as a new leptin-related mammary carcinogenesis model, we examined whether the number of terminal end buds of mammary gland, the serum biochemistry, leptin concentration in serum and adipose tissue are changed in 7-week-old female +/+, +/fa and *fa/fa* rats, and whether these changes and leptin, TNF- α and VEGF mRNA expression in adipose tissue of +/+ and +/fa rats are influenced by 10% corn oil diet for 5 weeks. We confirmed that mild hyperleptinemia was more pronounced in 7-week-old +/fa as compared with wild type (+/+) and hypoplasia of mammary glands characterized by fewer numbers of terminal end buds in *fa/fa* was not observed in +/fa. With 10% corn oil diet, leptin mRNA expression in adipose tissue showed increasing tendency both in +/fa and +/+. Comparing with +/+, adipose tissue in +/fa treated with 10% corn oil diet was found to be significantly increased in the concentration of leptin protein and tended to be elevated expression of TNF- α mRNA. These results suggest that +/fa with 10% corn oil diet may be a useful model for investigation of the participation of leptin and TNF- α in mammary gland carcinogenesis.

Key words: Leptin, Tumor models, Mammary cancer

INTRODUCTION

General obesity is an important risk factor of mammary cancer in postmenopausal women, and central obesity was further reported to increase mammary cancer risk in premenopausal as well as postmenopausal populations (Calle and Thun, 2004; Phillips *et al.*, 1996; Schaffler *et al.*, 2007). The mechanisms involved remain largely unclear, but it is suggested that various bioactive factors synthesized by adipose tissue might exert tumor-stimulatory effects on the mammary gland epithelium (Caldefie-Chezet *et al.*, 2005; Housa *et al.*, 2006). One principal bioactive substance produced by adipocytes is leptin (Anubhuti and Arora, 2008), a 167-aminoacid peptide hormone encoded by the obesity gene (*ob*), which is secreted and plays important roles in regulating food intake and energy expenditure through binding to specific receptors (OB-R) (Anubhuti and Arora, 2008). Lep-

tin also controls other common physiological processes such as immune responses, cell differentiation, proliferation and angiogenesis (Zhang *et al.*, 2005). Furthermore, several evidences suggest that leptin could be involved in tumorigenesis as a mitogenic, transforming or migration factor, especially active in the development of mammary, colorectal and prostate cancers (Garofalo and Surmacz, 2006; Hu *et al.*, 2002; Rouet-Benzineb *et al.*, 2004; Somasundar *et al.*, 2004).

Both leptin and OB-R appear to be significantly over-expressed in human mammary cancer tissue relative to non-cancer epithelium (Ishikawa *et al.*, 2004). In addition, higher expression of OB-R protein has been demonstrated in estrogen receptor α (ER α)-positive human mammary carcinoma cells MCF-7 and T47D than ER α -negative carcinoma cells MDA-MB-231 and MDA-MB 435 (Garofalo *et al.*, 2004). Leptin stimulates estrogen production through enhanced aromatase mRNA expres-

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sion, protein content and enzymatic activity in MCF-7, via AP-1 (Catalano *et al.*, 2003). In general, elevated lifetime estrogen exposure is considered a major risk factor for mammary cancer in human (Key *et al.*, 2002). Leptin signaling is also reported to play an important role in the growth of mammary cancers through promotion of the expression of vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor type 2 (VEGFR2) (Rene Gonzalez *et al.*, 2009). Moreover, its synthesis is influenced most notably by tumor necrosis factor- α (TNF- α) (Zhang *et al.*, 2000), insulin (Cusin *et al.*, 1995) and reproductive hormones (Machinal-Quelin *et al.*, 2002), all of which have been associated with mammary gland neoplastic processes. For example, there is evidence that hyperinsulinemia promotes mammary cancer progression through leptin-dependent mechanisms (Bartella *et al.*, 2008; Garofalo *et al.*, 2006). Estrogen regulates leptin productions in rats and humans subjects *in vivo* (Alonso *et al.*, 2007; Shimizu *et al.*, 1997).

TNF- α is a multifunctional cytokine that plays important roles in diverse cellular events such as immune function, cell survival, proliferation, differentiation, and death (Wang and Lin, 2008). Administration of TNF- α increased leptin mRNA and protein levels in adipose tissue of hamsters (Grunfeld *et al.*, 1996). Adipose tissues of the obese *db/db*, *ob/ob*, *tub/tub* mice, and the *fa/fa* Zucker rat expressed high levels of TNF- α mRNA and circulating plasma levels of TNF- α protein significantly elevated in *db/db* mice (Hotamisligil *et al.*, 1993). TNF- α , a proinflammatory cytokine, has been shown to be synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003), which may be involved in inflammation-associated carcinogenesis (Balkwill, 2009).

In animal models, a higher body weight is linked with increased incidences of both spontaneous and chemically induced mammary tumors (Haseman *et al.*, 1994; Waxler *et al.*, 1953; Wolff *et al.*, 1982). Zucker rats with a homogeneous spontaneous mutation in the leptin receptor gene (*fa/fa*) (Phillips *et al.*, 1996) are known to be obese, hyperphagic and hyperinsulinemic (Bray, 1977). In contrast, lean Zucker (*+fa* or *+/+*) rats show almost normal metabolic functions, and have been used as controls in various types of physiochemical and pathological experiments (Bray, 1977). The Zucker rat has been recognized as a superior model to investigate effects of obesity on chronic disease development, including cancer (Bray, 1977; de Assis *et al.*, 2006; Hakkak *et al.*, 2007), but its utility for investigations of mammary carcinogenesis is limited due to scant epithelial development in

mature mammary glands of obese as compared with lean counterparts (Hu *et al.*, 2002). Since it was reported that young heterozygous lean Zucker (*+fa*) rats demonstrate a number of differences from wild type lean Zucker (*+/+*) rats, e.g., higher body weights, fat cell size, inguinal fat pad weights, pad-to-body weight ratios, serum cholesterol, adipose tissue lipoprotein lipase and glycerol-3-phosphate dehydrogenase, hepatic and adipose tissue 6-phosphogluconate dehydrogenase activities and serum leptin levels (1.6 and 0.9 ng/ml in *+fa* and *+/+*, respectively, (Cleary and Phillips, 1999)) (Cleary *et al.*, 1999; Heo *et al.*, 2002; Phillips and Cleary, 1994; Truett *et al.*, 1995; Zhang *et al.*, 1997), we here investigated whether they might provide the basis for a leptin-related mammary carcinogenesis model. Two independent experiments were performed. In experiment 1, serum biochemistry, histological characteristics of mammary glands and leptin levels of serum and adipose tissue in 7-week-old female *+fa* lean Zucker rats were compared with those of *fa/fa* and *+/+* siblings. In experiment 2, we tested whether 10% corn oil diet affects serum biochemistry and histological characteristics of mammary glands as well as leptin, TNF- α and VEGF mRNA expression in adipose tissue of female *+fa* and *+/+* lean Zucker rats.

MATERIALS AND METHODS

Animals

Homozygous obese (*fa/fa*), heterozygous lean (*+fa*) and wild type (*+/+*) female Zucker rats at 6 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). They were housed in clear polycarbonate cages with heat-treated white wood chips for bedding (Sankyo Laboratory Service, Tokyo, Japan) in an air conditioned room ($24 \pm 1^\circ\text{C}$, $55 \pm 5\%$ relative humidity, 12 hr light and dark cycle) and given basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. The composition of the basal diet is 22.4% crude protein, 5.7% crude fat, 6.6% crude ash, 3.1% crude fiber, 7.8% moisture content and 54.5% nitrogen-free extract and the calorie of cereal-based diet is 359 kcal/100 g. The present study design was approved by the Animals Care and Utilization Committee of the National Institute of Health Sciences.

Genotyping

The animals were divided into each genotype group on the basis of genotyping, as described previously (Phillips *et al.*, 1996). For polymerase chain reaction (PCR) amplification of DNA sequences encoding leptin receptor isoform, digested 0.5 mm tail samples were amplified with the primers 5'-GTTTGGTATGGAAGTCACAG-3' and

5'-ACCAGCAGAGATGTATCCGAG3' at the annealing temperature of 67°C for 30 cycles. The PCR products were incubated with *Msp*I for 1 hr at 37°C to indicate the presence of the mutation-derived restriction site in Zucker rat genomic DNA.

Experiment 1

Female Zucker rats (+/+, *n* = 8; +/*fa*, *n* = 16; *fa/fa*, *n* = 6) at 7-weeks of age were weighed and sacrificed without overnight fasting and blood samples were collected from the abdominal aorta under ether anesthesia for serum biochemistry and leptin and insulin enzyme assays. Serum biochemistry measurements of glucose, triglyceride (TG), total cholesterol (T-Cho) and a double antibody radioimmunoassay for estradiol were performed at SRL (Tokyo, Japan). Leptin levels in serum and homogenates of adipose tissue that carefully excluded their mammary gland from the one side of an inguinal fat pad and serum insulin levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit, YK050 (Yanaihar Institute, Shizuoka, Japan) and by rat insulin ELISA (Mercodia AB, Uppsala, Sweden), respectively, according to the manufacturer's instructions. After macroscopic observation of abdominal viscera, subcutis and inguinal fat pads, liver and remaining inguinal fat pads containing mammary gland tissue were removed and fixed in 10% neutral buffered formalin for routine preparation of paraffin-embedded sections, then, hematoxylin and eosin (H.E.) staining and immunohistochemical analysis were performed. Livers were weighed before processing. The other side of inguinal fat pad in each animal was used for whole-mount preparation.

Experiment 2

Female Zucker rats at 7-weeks of age were fed basal (+/+, *n* = 5; +/*fa*, *n* = 6) or basal diet + 10% corn oil (+/+, *n* = 14; +/*fa*, *n* = 15) for 5 weeks and then sacrificed without overnight fasting in the same manner with experimental 1. In addition to the measured items in experiment 1 except serum estradiol concentration and total number of TEB in whole-mount preparation, expression of mRNAs for leptin, tumor necrosis factor- α (TNF- α), vascular endothelial growth factor A (VEGFA) and aromatase in inguinal adipose tissue was analyzed using real-time reverse transcription (RT)-PCR.

Mammary gland whole mounts and quantification

The whole-mount preparation protocol was a modification of previously described procedures (You *et al.*, 2002). Freshly dissected inguinal fat pad containing mammary glands tissues were placed flat between a pair of glass

slides and fixed in 10% formalin for 24 hr, then dehydrated with 70, 95 and 100% ethanol (about 3 hr in each). The samples were defatted in acetone for approximately 12 hr and rehydrated in diluted ethanol solution ranging from 100 to 40% for 3 hr each. The samples were stained with 0.005% toluidine blue for 30 min and then dehydrated again in ethanol solution. The tissue pieces were then finally immersed in xylene for approximately 6 hr and mounted on glass slides with a mounting agent. The total numbers of terminal end buds (TEB) were counted from the distal portions of the mammary gland under a stereomicroscope, according to the criteria of Russo (Russo *et al.*, 1990).

Immunohistochemistry

The streptavidin-biotin peroxidase complex method (StreptABComplex/HRP, DAKO, Glostrup, Denmark) was used to determine the expression and localization of leptin and leptin receptors in mammary glands and inguinal adipose tissue of Zucker rats at 7 and 12 weeks of age. Polyclonal antibodies against leptin (Ob) were purchased from Santa Cruz Biotechnology (A-20; Santa Cruz, CA, USA) and used at a dilution of 1/100. A polyclonal antibody against the leptin receptor (OB-R) recognizing both wild and mutant forms was accessed from Neuromics Antibodies (Edina, MN, USA) and used at 1/1000. Antigen retrieval was performed in an autoclave for 10 min at 121°C in 10 mM citrate buffer (pH 6.0) for leptin receptors. Sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibodies were included for each antigen using serial sections.

Real-time RT-PCR

Quantitative real-time RT-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems Japan, Tokyo, Japan) was performed for leptin (*Lep*), TNF- α (*Tnf*), VEGFA (*Vegfa*) and aromatase (*Cyp19a1*). One microgram aliquots of total RNA isolated from inguinal fat pads of all rats and from ovaries and livers of basal diet +/+ as positive and negative control of aromatase, respectively, in experiment 2 using Isogen™ (Nippon Gene, Tokyo, Japan) were applied to RT with a High-Capacity cDNA Archive Kit (Applied Biosystems Japan, Tokyo, Japan) in a 100 μ l total reaction volume. For real-time PCR analysis, ABI Assays-on-Demand™ TaqMan probe and primer sets from Applied Biosystems (available at <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267/>) were employed. Real-time PCR was performed in a 50- μ l reaction volume using the TaqMan probe detection

system (Applied Biosystems Japan) with specific primers, the corresponding TaqMan™ MGB probes (FAM™ dye labeled) and RT products. For the quantification of expression data, a standard curve method and normalization with a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase were applied.

Statistical analysis

Variance in data was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance for homogeneity (ANOVA) was used. In the heterogeneous cases, the Kruskal Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between groups; in experiment 1, among all groups; in experiment 2, +/+ basal diet vs. +/+ 10% corn oil, +/+ basal diet vs. +/fa basal diet, +/+ 10% corn oil vs. +/fa 10% corn oil and +/fa basal diet vs. +/fa 10% corn oil. Value are presented as means $\pm$ standard deviations or standard error. *p* values of less than 0.05 were considered to be statistically significant.

RESULTS

Experiment 1

In female fatty (*fa/fa*) Zucker rats at 7-weeks old of age, body weights and absolute and relative liver weights

were higher ($p < 0.05$ or 0.01) than those of lean +/+ and/or +/fa rats (Table 1) and excess accumulation of adipose tissue in abdominal viscera, subcutis and inguinal fat pads at necropsy and increased storage of hepatocellular glycogen on histopathology were observed. There were higher ($p < 0.05$ or 0.01) concentrations of serum TG, T-Chol and insulin in *fa/fa* rats than in +/+ and/or +/fa rats, but not of glucose and estradiol (Table 2). No difference of body and liver weights and serum T-Chol, TG and insulin values were observed between +/+ and +/fa rats. Leptin concentrations in serum and adipose tissue were higher ($p < 0.05$ or 0.01) in *fa/fa* rats than +/+ and +/fa rats, and those of serum were also higher in +/fa rats ($p < 0.05$) than in +/+ rats (Table 3).

With inguinal mammary gland whole mounts, poorly developed tissue characterized by thinner ducts and immature glands and lower ($p < 0.01$) numbers of TEB (Fig. 1) was observed in female fatty (*fa/fa*) Zucker rats at 7-week old of age, as compared with lean +/+ and +/fa rats. Immunohistochemical analysis revealed that adipocytes in inguinal fat pad express leptin and its intensity was increased in hypertrophied adipocytes of *fa/fa* rats as compared with +/+ and +/fa (data not shown). Ductal and glandular epithelium of mammary gland of all genotypes showed positive reaction to an anti-leptin receptor antibody that recognized both wild and mutant form (data not shown).

Table 1. Experiment 1; body and liver weights

Genotype	N	Body weight (g)	Absolute liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	8	152 $\pm$ 3 ^a	7.1 $\pm$ 0.4	4.7 $\pm$ 0.2
+/fa	16	153 $\pm$ 8	7.2 $\pm$ 1.0	4.7 $\pm$ 0.5
fa/fa	6	222 $\pm$ 9 ^{*,##}	11.5 $\pm$ 1.3 ^{*,##}	5.2 $\pm$ 0.4 [#]

N: No. of animals. ^a: Mean $\pm$ S.D.

^{**}: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test).

^{*,##}: Significantly different from +/fa at $p < 0.05$ and 0.01 , respectively (Dunnett's multiple test).

Table 2. Experiment 1; serum biochemistry

Genotype	N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)	Estradiol (pg/ml)
+/+	8	142 $\pm$ 7 ^a	153 $\pm$ 44	90 $\pm$ 11	1.2 $\pm$ 0.3	34 $\pm$ 30 ^b
+/fa	16	149 $\pm$ 28	165 $\pm$ 50	87 $\pm$ 7	1.4 $\pm$ 0.6	24 $\pm$ 13 ^c
fa/fa	6	155 $\pm$ 40	302 $\pm$ 134 [#]	129 $\pm$ 14 ^{*,##}	6.8 $\pm$ 6.0 ^{*,##}	19 $\pm$ 10

N: No. of animals. ^a: Mean $\pm$ S.D. ^b: $n = 7$ ^c: $n = 15$

^{**}: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test)

^{*,##}: Significantly different from +/fa at $p < 0.05$ and 0.01 , respectively (Dunnett's multiple test)

+/*fa* Zucker as mammary carcinogenesis model

Fig. 1. Experiment 1; representative whole-mount preparation (a, toluidine blue staining, original magnification x40), histology (b, HE staining, original magnification x200) and numbers of terminal end buds (TEBs) (c) of mammary tissue of 7-week-old female Zucker rats. Poorly developed tissue characterized by thinner ducts and immature glands and lower numbers of TEB was noted in female fatty (*fa/fa*, $n = 6$) Zucker rats at 7-week old of age, comparing with +/+ ($n = 8$) and +/*fa* ($n = 16$) rats. **: Significantly different from +/+ at $p < 0.01$. #: Significantly different from +/*fa* at $p < 0.01$.

Table 3. Experiment 1; leptin levels in serum and adipose tissue

Genotype	N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	8	0.08 ± 0.02^a	1.3 ± 0.6
+/ <i>fa</i>	16	$0.14 \pm 0.05^*$	1.6 ± 1.0
<i>fa/fa</i>	6	$1.24 \pm 0.13^{*,\#}$	$8.5 \pm 1.6^{*,\#}$

N: No. of animals. *: Mean $\pm$ S.D.

*, **: Significantly different from +/+ at $p < 0.05$ and 0.01 , respectively (Dunnett's multiple test)

#, #: Significantly different from +/*fa* at $p < 0.05$ and 0.01 , respectively (Dunnett's multiple test)

Experiment 2

In +/*fa* rats significantly higher final body weight ($p < 0.01$) was shown in rats fed 10% corn oil when compared with rats fed basal diet (Table 4). In +/*fa* rats fed 10% corn oil, absolute and relative liver weight were significantly lower than those of +/+ ($p < 0.05$ or 0.01 , Table 4). No histopathological differences in liver and mammary glands were observed between +/+ and +/*fa* rats fed basal or 10% corn oil mixed diet for 5 weeks (data not shown). In serum biochemistry, glucose concentration ($p < 0.01$) showed significantly lower values in +/*fa* rats as compared to +/+, but TG showed lower values without statis-

Table 4. Experiment 2; final body and liver weights

Genotype and diet		N	Body weight (g)	Liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	Basal diet	5	229 ± 10 ^a	8.2 ± 1.0	3.6 ± 0.3
	10% Corn oil diet	6	233 ± 12	8.4 ± 0.7	3.6 ± 0.2
+/ <i>fa</i>	Basal diet	14	222 ± 10	7.7 ± 0.6	3.5 ± 0.2
	10% Corn oil diet	15	235 ± 9 ^{ss}	7.8 ± 0.2 ^{##}	3.3 ± 0.1 [#]

N: No. of animals. *: Mean ± S.D.

#, ##: Significantly different from +/+ 10% Corn oil diet at $p < 0.05$ and 0.01 , respectively (Dunnett's multiple test)ss: Significantly different from +/*fa* basal diet at $p < 0.01$ (Dunnett's multiple test)**Table 5.** Experiment 2; serum biochemistry

Genotype and diet		N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)
+/+	Basal diet	5	179 ± 24 ^a	346 ± 90	92 ± 20	1.9 ± 1.1
	10% Corn oil diet	6	172 ± 15	233 ± 77	98 ± 6	1.9 ± 0.8
+/ <i>fa</i>	Basal diet	14	153 ± 13 ^{**}	239 ± 100	86 ± 11	2.0 ± 0.9
	10% Corn oil diet	15	162 ± 15	176 ± 85	84 ± 10	1.7 ± 0.9

N: No. of animals. *: Mean ± S.D.

: Significantly different from +/+ basal diet at $p < 0.01$ (Dunnett's multiple test)Table 6.** Experiment 2; leptin levels in serum and adipose tissue

Genotype and diet		N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	Basal diet	5	0.2 ± 0.1 ^a	3.8 ± 1.0
	10% Corn oil diet	6	0.5 ± 0.3	2.6 ± 0.7
+/ <i>fa</i>	Basal diet	14	0.3 ± 0.2	4.7 ± 2.2
	10% Corn oil diet	15	0.5 ± 0.3 [*]	7.7 ± 1.5 ^{*,##}

N: No. of animals. *: Mean ± S.D.

*: Significantly different from +/*fa* basal diet at $p < 0.05$ (Dunnett's multiple test)##: Significantly different from +/+ 10% Corn oil diet at $p < 0.01$ (Dunnett's multiple test)

tical significance in +/*fa* rats with and without 10% corn oil mixed feeding as compared to their +/+ counterparts (Table 5). Insulin concentrations showed similar values among all the groups, but T-Chol concentration showed lower in +/*fa* than +/+ with 10% corn oil feeding (Table 5). Without 10% corn oil feeding, serum and adipose tissue leptin levels in +/*fa* showed a non-significant tendency for elevation than in +/+. With 10% corn oil feeding, serum and adipose tissue leptin levels were significantly increased in +/*fa* than in +/+ with basal diet ($p < 0.05$). Moreover, 10% corn oil feeding increased adipose tissue leptin level in +/*fa* than in +/+ ($p < 0.01$, Table 6).

With real-time RT-PCR analysis in adipose tissue, increased tendencies for leptin and TNF- α mRNA expres-

sion and opposite decrease of VEGFA were observed with the 10% corn oil diet in both genotypes (Fig. 2). Regarding TNF- α mRNA expression, +/*fa* rats showed higher tendencies regardless of the diet (Fig. 2b). Expression of aromatase mRNA was detected in ovaries as a positive control, but not in adipose tissue and livers of either +/*fa* and +/+ rats (data not shown).

DISCUSSION

The present investigation of female heterozygous lean Zucker rats in comparison with *fa/fa* and +/+ animals pointed to the potential of heterozygous lean Zucker rat as a possible new leptin-related mammary carcinogenesis

+/-fa Zucker as mammary carcinogenesis model

Fig. 2. Experiment 2; leptin (a), TNF- α (b) and VEGFA (c) mRNA expression in adipose tissue of female Zucker rats fed 10% corn oil for 5 weeks. Tendencies for slight increase of leptin and TNF- α expression and decrease of VEGFA were observed on feeding the 10% corn oil diet with both genotypes, but TNF- α expression showed higher tendencies in *+/-fa* rats regardless of the diet. mRNA expression was normalized to the expression level of a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase. Basal diet = open bar, 10% corn oil = closed bar. $n = 3-5$ (basal diet, *+/+*), $4-6$ (10% corn oil, *+/+*), $10-14$ (basal diet, *+/-fa*), $14-15$ (10% corn oil, *+/-fa*). Values were set at 1 in *+/+* basal diet group and expressed as mean $\pm$ S.E. relative values.

model. While fatty *fa/fa* rats show dramatically high values for serum insulin, and serum and adipose tissue leptin, they have only few TEBs at around 7 weeks of age, when rats are reported to be most sensitive to carcinogens targeting the mammary gland (Russo *et al.*, 1979). In contrast, lean *+/-fa* rats feature normal mammary gland development. Corn oil-supplemented diet increased the serum leptin level. Interestingly, the increase of the leptin level in adipose tissue by 10% corn-oil diet was only observed in *+/-fa* rat. TNF- α mRNA expression in *+/-fa* was higher than in *+/+* and further increased with corn-oil diet. All these results suggested female lean *+/-fa* rats may be a potential model for investigation of mammary carcinogenesis in which leptin and TNF- α are the major related factors.

Epidemiologically mammary cancer has been shown to be associated with obesity in postmenopausal women (Calle and Thun, 2004) and hyperleptinemia is also recognized as a risk factor (Wu *et al.*, 2009). Fatty Zucker rats with hyperleptinemia have been widely used as animal obesity model which mimics human obesity and the metabolic syndrome, and recently mammary gland carcinogenicity was investigated with 7,12-dimethylbenz(a)anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU)-treated Zucker rat models. Hakkak *et al.* (2005, 2007) reported that DMBA administration by gavage at 50 days old at 65 mg/kg body weight caused more mammary tumors in female obese Zucker (*fa/fa*) rats than their lean (*+/-fa* or *+/+*) siblings. In this model, it is uncertain

which obese-related parameters, e.g., hyperinsulinemia, hyperleptinemia or hyperlipidemia, affected the mammary carcinogenesis. In contrast, Lee *et al.* (2001) indicated that no increase in susceptibility with MNU at doses of 37.5 or 20 mg/kg body weight administered to fifty-day-old female lean (*+/-fa* or *+/+*) or obese Zucker (*fa/fa*) rats. The controversial results may be partially due to the dose of administered carcinogen based on the body weight and poor development of mammary glands with low numbers of TEBs in homozygous obese Zucker rats, as shown in Fig. 1. Scant epithelial development in mammary glands were also known in non-transgenic genetically obese leptin-deficient (*ob/ob*) and genetically obese leptin receptor-deficient (*db/db*) mice as compared with their lean counterparts (Hu *et al.*, 2002). Impaired development of mammary glands have been described in transgenic *TGF- α /ob/ob* mice (Cleary *et al.*, 2003) and high fat diet-dependent nulliparous nonpregnant obese mice (Kamikawa *et al.*, 2009). Leptin-dependent inhibition of cell proliferation has been reported in noncancerous mouse mammary epithelial cell line (Baratta *et al.*, 2003; Motta *et al.*, 2007).

It has been also reported that the reason for the poorly developed mammary gland in obese might be abnormal endogenous steroid production rather than hyperleptinemia (Marin Bivens and Olster, 1997). *fa/fa* Zucker rats also show delayed vaginal opening, subsequent abnormal estrous cyclicity, undeveloped uteri and lack of deciduomata formation (Saiduddin *et al.*, 1973) as well as

abnormal estrous cycles (Marin Bivens and Olster, 1997). These facts suggest that young *fa/fa* Zucker rats may have disadvantage as a mammary carcinogenesis model in aspects of abnormal development of mammary glands and hormone environment. Therefore, the model in which the level of leptin can be effectively controlled by some exogenous factor such as diet might be a better one. In the present study, the increase of adipose tissue leptin by corn-oil diet was more evident in *+/fa* than *+/+*. Maher *et al.* (1996) also showed that adipose tissue leptin levels was significantly higher in fat pads of *+/fa* compared to wild type rats and *+/fa* rats fed high-fat diet showed an additional two-fold increase in leptin levels compared to wild type rats on the same diet.

It was reported that adipose tissues of the obese *fa/fa* Zucker rat expressed high level of TNF- α mRNA as compared to lean *+/+* or *+/fa* (Hotamisligil *et al.*, 1993). In the present study expression of TNF- α mRNA in the adipose tissue tended to be higher in *+/fa* Zucker rats than *+/+* and further increased by the 10% corn oil diet, but there was no statistical significances presumably due to small number of *+/+* and/or wide variability. TNF- α stimulates the release of preformed leptin from human mature adipocytes and differentiated preadipocytes (Zhang *et al.*, 2000), and has the ability to promote tumor progression and cancer cell dissemination (Montesano *et al.*, 2005). TNF- α is synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003). Mammary glands of a diet induced obese mouse model harbored more infiltrating macrophages (Kamikawa *et al.*, 2009), while in the present study, increased macrophage infiltration was not observed in *+/fa* with or without the 10% corn oil diet (data not shown). Taken together, our results suggest that TNF- α expression may be stimulated by leptin-rich adipocyte rather than macrophage around mammary glands in *+/fa* and further by the 10% corn oil diet to promote leptin secretion and mammary carcinogenesis. There were no significant differences in VEGFA mRNA expression adipose tissue between our *+/fa* and *+/+* rats and links with leptin gene expression are not clear (Hausman and Richardson, 2004).

Expression level of leptin mRNA in adipose tissue of 12-week-old *+/fa* rats did not show statistically significant differences from those of *+/+* rats (Fig. 2a), while that of leptin protein in adipose tissue of *+/fa* fed with 10% corn oil was significantly higher than those of *+/+* rats (Table 6). Our present data suggested that differences in translation efficiency, stability and efficient usage of leptin protein might be related to inconsistency of leptin mRNA expression and protein levels in *+/fa* rats. In addition, the adi-

pose tissue leptin level in 12 week-old females *+/fa* is over 3 times higher than those of 7-week-old *+/+* in the present study. A previous study indicated that adipose tissue mRNA levels for leptin were higher in *+/fa* rats than *+/+* rats at 10-days of age (Zhang *et al.*, 1997). Leptin level in serum and leptin mRNA expression in adipose tissue in Wistar rats was increased with age (Oliver *et al.*, 2001).

Fasting serum glucose, TG and insulin were not changed in *+/fa* compared to *+/+* in many studies (Phillips and Cleary, 1994; Schwarzer *et al.*, 1997; Zhang *et al.*, 1997). Glucose and TG concentration showed lower values with and without statistical significance, respectively, in experiment 2, but not in experiment 1. Conflicting results may be partially explained by that animals were sacrificed without overnight fasting, because serum glucose and TG levels are easily affected by food consumption. On the other hand, T-cho concentration showed lower in *+/fa* than *+/+* with 10% corn oil, which may be due to cholesterol elimination promoted by increased leptin (VanPatten *et al.*, 2001). Period of 5 weeks for diet fat supplementation is apparently shorter than that necessary for mammary carcinogenesis, but it is considered enough to examine the effects of high fat diet on the factors related to mammary carcinogenesis (Flachs *et al.*, 2006).

In conclusion, these results suggests that *+/fa* rats may be a useful model for investigation of mammary carcinogenesis in which leptin and TNF- α are the major related factors.

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