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Phenotypic Characterization of *Ggt1*^{dwg/dwg} Mice, a Mouse Model for Hereditary γ -Glutamyl Transferase Deficiency

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Abstract: *Ggt1*^{dwg/dwg} mice are spontaneous mutant mice with a nucleotide deletion in the *Ggt1* gene. They are characterized by dwarfism, cataract, and coat color abnormality. These abnormalities in the external appearance of *Ggt1*^{dwg/dwg} mice closely resemble those of previously reported GGT1-deficient mice, *Ggt1*^{tm1Zuk/tm1Zuk} (*Ggt1*^{–/–}) and *Ggt1*^{enu1/enu1}, generated by gene targeting or ENU mutagenesis. However, whether the pathological features of *Ggt1*^{dwg/dwg} mice are also similar to those of the *Ggt1*^{–/–} and *Ggt1*^{enu1/enu1} mice remains unclear. To clarify the pathogenesis of *Ggt1*^{dwg/dwg} mice, we physiologically and histologically investigated the abnormalities of *Ggt1*^{dwg/dwg} mice in this study. First, we analyzed the activity of GGT1 and GSH levels in *Ggt1*^{dwg/dwg} mice. GGT1 activity in the *Ggt1*^{dwg/dwg} mice was reduced to approximately 4.0% of that in the wild-type mice. Plasma and kidney GSH levels were markedly increased, while eye and liver GSH levels were markedly decreased, in the *Ggt1*^{dwg/dwg} mice. Notably, no significant difference in survival rate was observed between the *Ggt1*^{dwg/dwg} and wild-type mice, whereas high mortality was reported in the *Ggt1*^{–/–} and *Ggt1*^{enu1/enu1} mice. Growth retardation, degeneration of lens fibers, and an increased number of osteoclasts in the *Ggt1*^{dwg/dwg} mice were reversed by administration of *N*-acetyl-L-cysteine, a precursor of GSH synthesis. Thus, we conclude that the abnormalities of *Ggt1*^{dwg/dwg} mice are caused by alteration of the GSH levels due to the depression of GGT1 activity and that *Ggt1*^{dwg/dwg} mice will be a useful model for GGT deficiency with peculiar features.

Key words: *ggt1*, glutathione, mouse, mutation

Introduction

γ -Glutamyl transferase (GGT) is a cell surface heterodimeric glycoprotein that consists of a heavy-chain domain with an anchor sequence and a light-chain domain with activity for the transfer of the γ -glutamyl moiety of glutathione (GSH) or GSH conjugates to an appropriate acceptor [13, 18]. GGT is most abundant on the luminal surface of the proximal tubule of the kidney [4] and plays an important role for the recovery of cysteine from urinary GSH in the kidney [14].

Extracellular GSH is cleaved by GGT into a γ -glutamyl moiety and cysteinylglycine, which is further degraded into cysteine and glycine and transported inside cells to be used for the intracellular resynthesis of GSH through the γ -glutamyl cycle. GSH is crucial for the protection of cells against oxidative stress and other forms of cellular injury resulting from cytotoxic and carcinogenic xenobiotic compounds. Therefore, GGT has been considered to be an integral factor for antioxidant defense and de-

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toxification systems through the contribution to GSH homeostasis.

The GGT family comprises 13 types of GGT in humans and four types in mice and rats, of which only two types, GGT1 and GGT5, have been confirmed to be able to cleave the γ -glutamyl moiety [7, 20]. Among the GGT family, GGT1 is the most well-investigated enzyme using the mouse with a genetically modified *Ggt1* gene. Mice with targeted disruption of the *Ggt1* gene (*Ggt1^{tm1Zuk}*) and those with chemically induced [*N*-ethyl-*N*-nitrosourea (ENU)-induced] mutation, referred to as *Ggt1^{-/-}* mice and *Ggt1^{enu1/enu1}* mice in this paper, respectively, have been developed. Dwarfism, cataracts, infertility, and early death have been shown to be common phenotypes in these mice [5, 10, 14]. Glutathionemia, glutathionuria, deficiency of cellular GSH, and high susceptibility to oxidative stress have also been reported in these mice [1, 5, 14]. These abnormalities are reversed by administration of *N*-acetyl-*L*-cysteine (NAC), a precursor of GSH synthesis [2, 11, 12, 14]. These findings suggest that GGT1 is essential for GSH homeostasis and is associated with regulation of the cellular antioxidant defense system. In human hereditary GGT deficiency, mental retardation, abnormal behavior, and absence seizure have been observed in addition to glutathionemia and glutathionuria [3, 8], but the association of GGT deficiency with these symptoms remains unclear. Therefore, animal models with depression of GGT activity are an exquisitely powerful tool for studying the disorders related to the physiological functions of GGT.

Ggt1^{dwg/dwg} mice are spontaneous mutant mice characterized by dwarfism, cataracts, gray coats, infertility, and an increased number of osteoclasts [6]. Reduced numbers of chondrocytes in tibial growth plates have also been reported in *Ggt1^{dwg/dwg}* mice [19]. These phenotypic features of *Ggt1^{dwg/dwg}* mice are similar to those of *Ggt1^{-/-}* and *Ggt1^{enu1/enu1}* mice [11, 12, 14], and *Ggt1^{dwg/dwg}* mice have been found to contain a deletion of 13 nucleotides in exon 7 of the *Ggt1* gene [19]. These findings suggest that along with the *Ggt1^{-/-}* mice and *Ggt1^{enu1/enu1}* mice, *Ggt1^{dwg/dwg}* mice could be a mouse model for GGT deficiency; however, there are no precise data regarding the pathological abnormalities in *Ggt1^{dwg/dwg}* mice. Therefore, in the present study, we investigated the pathophysiological and pathohistological properties of *Ggt1^{dwg/dwg}* mice to clarify the utility of *Ggt1^{dwg/dwg}* mice as models for GGT deficiency.

Materials and Methods

Mice

Heterozygous (*Ggt1^{dwg/+}*) mutant mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and the strain was maintained by sib mating of heterozygotes. Genotyping of the mice was performed by polymerase chain reaction (PCR) using genomic DNA, as described previously [19]. The mice were maintained on a 12-h light/dark cycle and provided food and water *ad libitum*. The body length (naso-anal) and weight of mice were measured every week. For comparison of the survival rate, Kaplan–Meier survival analysis was used to determine statistical significance of the differences between the different genotypes of mice. All the animal experiments were approved by the Animal Committee of Okayama University and were conducted in accordance with the Guidelines for Animal Experiments at Okayama University.

Measurement of GGT activity

GGT activity was determined following the method of Orlowski and Meister [16]. The mice were euthanized at 10–11 weeks of age. Their kidneys were collected, immediately washed with phosphate-buffered saline (PBS), and homogenized in 10% w/v of 0.1 M Tris-HCl (pH 9.0). The homogenized samples were kept on ice or stored at -20°C until measurement. GGT activities were determined by calculation of the molar absorption ratio of *p*-nitroaniline at 410 nm ($\epsilon=8.8\text{ mM}^{-1}\text{ cm}^{-1}$) and expressed in nanomoles of *p*-nitroaniline produced/min/mg of protein. Protein concentrations were determined using the bicinchoninic acid method (Sigma-Aldrich, St. Louis, MO, USA).

Measurement of GSH

The kidneys, liver, and eyes were collected from the mice at 10–11 weeks of age. Plasma was separated by centrifugation of blood collected from the caudal vena cava. The collected samples were stored at -80°C until measurement. Tissues were homogenized in ice-cold 5% metaphosphoric acid solution. After centrifugation at $1,000 \times g$ for 10 min at 4°C , the upper clear aqueous phase was collected and used to determine the concentration of total GSH using a GSH/GSSG Cuvette Assay Kit (Oxford Biomedical Research, Rochester Hills, MI, USA).

Histological analysis

The eyes of the mice were fixed in Bouin's solution for at least 16 h at room temperature. The specimens were dehydrated in ethanol, embedded in paraffin, and sectioned at 5 μ m. The sections were stained with hematoxylin and eosin. Tibiae were fixed in 4% paraformaldehyde overnight at 4°C and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 14 days. To determine the number of osteoclasts, sections of tibiae were stained using a tartrate-resistant acid phosphatase/alkaline phosphatase (TRAP/ALP) stain kit (Wako, Tokyo, Japan). The numbers of TRAP-positive osteoclasts per millimeter at the bone adjacent to the trabecular surface were counted under a microscope.

NAC administration

The *Ggt1^{dwg/dwg}* and wild-type mice were supplied with NAC dissolved in drinking water (10 g/l) from 3 weeks to 9 weeks of age. During the experiment, body length and weight were measured every week, and the eyes and tibiae were collected after administration for 6 weeks.

Statistical analysis

The unpaired *t*-test or the log-rank test was used for statistical analysis. A *P*-value <0.05 was considered statistically significant.

Results

GGT activities and GSH levels in *Ggt1^{dwg/dwg}* mice

To clarify the influence of *Ggt1^{dwg}* mutation on GGT1 function, we assayed the activity of GGT with the extracts of whole kidneys from the *Ggt1^{dwg/dwg}*, *Ggt1^{dwg/+}*, and wild-type (*Ggt1^{+/+}*) mice (Table 1). High activity of GGT was detected in the wild-type mice as previously reported [9, 14]. In contrast, GGT activity in the *Ggt1^{dwg/+}* mice was significantly decreased (72.5% of that in the

Table 1. GGT activity in the kidney of wild-type, *Ggt1^{dwg/+}*, and *Ggt1^{dwg/dwg}* mice

Wild-type	<i>Ggt1^{dwg/+}</i>	<i>Ggt1^{dwg/dwg}</i>
1012.96 $\pm$ 52.81 ^{a)} (n=4)	733.95 $\pm$ 47.66 ^{b)} (n=4)	40.96 $\pm$ 12.20 ^{c)} (n=3)

Activity is defined as nanomoles *p*-nitroaniline produced/mg protein/min. Data are presented as mean concentrations with the SD. ^{a, b, c)}Different superscripts indicate significant differences in treatment (*P*<0.05).

wild-type mice), and a dramatic decrease in activity (4.0% of that in the wild-type mice) was observed in the *Ggt1^{dwg/dwg}* mice (Table 1).

GSH levels in *Ggt1^{dwg/dwg}* mice

To further assess the influence of the decrease in GGT activity on GSH metabolism, we measured GSH levels in the plasma, kidney, liver, and eye of the *Ggt1^{dwg/dwg}* and wild-type (*Ggt1^{+/+}*) mice. Plasma GSH levels in the *Ggt1^{dwg/dwg}* mice were approximately 2.6-fold higher compared with those in the wild-type mice. Liver and eye GSH levels in the *Ggt1^{dwg/dwg}* mice were markedly decreased compared with those in the wild-type (*Ggt1^{+/+}*) mice (27 and 15%, respectively, of those in the wild-type mice) (Table 2). In contrast, kidney GSH levels in the *Ggt1^{dwg/dwg}* mice were markedly higher than those in the wild-type mice (Table 2).

Phenotypic features of *Ggt1^{dwg/dwg}* mice

To clarify the features of the *Ggt1^{dwg/dwg}* mice in terms of body growth, we measured the body length and weight of the *Ggt1^{dwg/dwg}* and wild-type mice. Although the *Ggt1^{dwg/dwg}* mice were indistinguishable from their normal littermates in appearance at birth, a difference in the body length and weight appeared by 3 weeks of age (Fig. 1A and 1B). The growth of the *Ggt1^{dwg/dwg}* mice from 3 weeks to 9 weeks of age was apparently slow, and the body length and weight at 9 weeks of age were approx-

Table 2. GSH levels in wild-type and *Ggt1^{dwg/dwg}* mice

	Wild-type	<i>Ggt1^{dwg/dwg}</i>	% of wild-type
Plasma, μ M	151 $\pm$ 92.3	399 $\pm$ 37.2*	264
Kidney, μ mol/g tissue	0.17 $\pm$ 0.06	17.3 $\pm$ 3.00**	10,176
Liver, μ mol/g tissue	27.4 $\pm$ 3.49	7.47 $\pm$ 1.01***	27.3
Eye, μ mol/g tissue	8.85 $\pm$ 0.57	1.35 $\pm$ 0.18***	15.3

Three male mice were used in each group. Data are presented as mean concentrations $\pm$ SD. **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. 1. Growth of wild-type, *Ggt1^{dwg/dwg}*, and NAC-treated *Ggt1^{dwg/dwg}* mice. (A) Body length of wild-type, *Ggt1^{dwg/dwg}*, and NAC-treated *Ggt1^{dwg/dwg}* mice from 3 weeks to 9 weeks of age. (B) Body weight of wild-type, *Ggt1^{dwg/dwg}*, and NAC-treated *Ggt1^{dwg/dwg}* mice from 3 weeks to 9 weeks of age. (C) Gross appearance of wild-type, *Ggt1^{dwg/dwg}*, and NAC-treated *Ggt1^{dwg/dwg}* mice at 9 weeks of age. Each value in A and B represents a mean $\pm$ SD ($n=3$). * $P<0.05$ compared with the *Ggt1^{dwg/dwg}* group.

imately 83% and 56%, respectively, of those of their wild-type littermates (Fig. 1A–C).

At 3 weeks of age, lens opacities began to be recognized in the *Ggt1^{dwg/dwg}* mice by external observation. Histological analysis revealed the appearance of small vacuoles near the lens epithelium in the *Ggt1^{dwg/dwg}* mice (Fig. 2A). At 9 weeks of age, the vacuoles were swollen around the nuclear and cortical regions, and most lens fibers were degenerated (Fig. 2B). No histological changes were observed in the lens of the wild-type and *Ggt1^{dwg/+}* mice at 3 and 9 weeks of age (Fig. 2C and D2). To examine the change in the number of osteoclasts, TRAP-positive osteoclasts in tibiae were counted in the *Ggt1^{dwg/dwg}* and wild-type mice. An approximately 1.4-fold increase was observed in the number of osteoclasts in the *Ggt1^{dwg/dwg}* mice compared with that in the wild-type mice (Table 3). Kaplan–Meier survival analysis showed that approximately 90% of the *Ggt1^{dwg/dwg}* mice survived until 25 weeks after birth, and no significant

difference in survival rate was found between the *Ggt1^{dwg/dwg}* and wild-type mice (Fig. 3). It should be noted that these results clearly contradicted the previous reports indicating that the survival rates of the *Ggt1^{-/-}* and *Ggt1^{enu1/enu1}* mice at 25 weeks of age are about 10 and 50%, respectively [5, 14].

NAC treatment in *Ggt1^{dwg/dwg}* mice

To elucidate whether phenotypic alterations in the *Ggt1^{dwg/dwg}* mice result from GSH deficiency caused by a mutation in the *Ggt1* gene, we investigated whether supplementation with NAC (a precursor of GSH synthesis) can rescue these phenotypic abnormalities of *Ggt1^{dwg/dwg}* mice. During administration for 6 weeks, the body length and weight of the NAC-treated *Ggt1^{dwg/dwg}* mice were significantly increased compared with those of the untreated *Ggt1^{dwg/dwg}* mice (Fig. 1A and 1B), although the body length and weight did not reach the size of the wild-type mice (Fig. 1A–C). With regard to the

Fig. 2. Histology of the eyes of wild-type and *Ggt1*^{dwg/dwg} mice. The eyes of *Ggt1*^{dwg/dwg} (A and B), wild-type (C and D), and NAC-treated *Ggt1*^{dwg/dwg} (E and F) mice. The eyes of *Ggt1*^{dwg/dwg} and wild-type mice were observed at 3 weeks (A and C) and 9 weeks (B and D) of age. The eyes of NAC-treated *Ggt1*^{dwg/dwg} mice were observed at 9 weeks of age (E and F). Scale bars (A–E): 500 μ m.

Table 3. Number of osteoclasts in wild-type, *Ggt1*^{dwg/dwg}, and NAC-treated *Ggt1*^{dwg/dwg} mice

	Wild-type	<i>Ggt1</i> ^{dwg/dwg}	NAC-treated <i>Ggt1</i> ^{dwg/dwg}
Osteoclast number (/mm)	4.31 $\pm$ 0.08	5.89 $\pm$ 0.61*	2.18 $\pm$ 0.23*

Three male mice were used in each group. Data are presented as mean values $\pm$ SD. * P <0.05 compared with the wild-type group.

Fig. 3. Survival rate of wild-type, *Ggt1*^{dwg/+}, and *Ggt1*^{dwg/dwg} mice. The cumulative survival rate was plotted against age in weeks. No significant difference was observed between the *Ggt1*^{dwg/dwg} group and the other two groups by analysis with the long-rank test.

development of cataracts, one of three NAC-treated *Ggt1*^{dwg/dwg} mice showed some degree of degeneration of lens fibers (Fig. 2E), which was less severe than the abnormalities observed in the lens of the untreated *Ggt1*^{dwg/dwg} mice, and no abnormalities were observed in the other two *Ggt1*^{dwg/dwg} mice (Fig. 2F). The increased number of osteoclasts in the *Ggt1*^{dwg/dwg} mice was also clearly reversed by NAC administration (Table 3). These data show that growth retardation, cataracts, and an increased number of osteoclasts in *Ggt1*^{dwg/dwg} mice are completely or partially reversed by NAC treatment and suggest that the abnormalities in *Ggt1*^{dwg/dwg} mice are caused by GSH deficiency.

Discussion

Ggt1^{dwg} is a spontaneous mutation caused by a 13 bp deletion in exon 7 of the *Ggt1* gene [19]. Therefore, mice homozygous for *Ggt1*^{dwg} are expected to be a useful model for analyzing the physiological role of GGT1 as

other mouse models with the genetically modified *Ggtl* gene [5, 14]. In the present study, to clarify the characteristic of this mutant mouse as a model for GGT deficiency, we investigated the physiological and histological phenotypes of *Ggtl*^{dwg/dwg} mice.

Measurement of GGT activity in the kidney revealed that mice heterozygous or homozygous for the *Ggtl*^{dwg} mutation showed significantly lower activity. In particular, the *Ggtl*^{dwg/dwg} mice showed a remarkable decrease in activity (only 4.0% of the GGT activity in the wild-type mice). Because the GGT1 of *Ggtl*^{dwg/dwg} mice retains slight activity, unlike the GGT1 of *Ggtl*^{-/-} mice, which shows no activity, the *Ggtl*^{dwg} allele is concluded to be a hypomorphic mutation of the *Ggtl* gene. In the kidney of *Ggtl*^{dwg/dwg} mice, three types of transcripts encoding GGT1 lacking the light-chain domain, a transcript encoding GGT1 with an intact light-chain domain, and an internally deleted heavy-chain domain have been isolated [19]. Thus, the retention of slight GGT1 activity in the *Ggtl*^{dwg/dwg} mice might be accounted for by the existence of GGT1 with the light-chain domain, an important region for the activity.

We confirmed the increase in plasma GSH levels and decrease in liver and eye GSH levels in the *Ggtl*^{dwg/dwg} mice. These alterations of the GSH levels were almost the same as those previously reported for *Ggtl*^{-/-} and *Ggtl*^{enu1/enu1} mice [5, 14]. Interestingly, kidney GSH levels in the *Ggtl*^{dwg/dwg} mice were markedly increased despite the lower activity of GGT1 in the kidney, whereas kidney GSH levels in the *Ggtl*^{-/-} mice were significantly decreased. Residual GGT1 activity in the *Ggtl*^{dwg/dwg} mice may be related to these opposite findings because an increase in kidney GSH levels has been found in *Ggtl*^{enu1/enu1} mice, which also show slight GGT activity [5].

By phenotypic analyses, we revealed that the *Ggtl*^{dwg/dwg} mice had growth retardation, vacuolar degeneration of lens fibers, and an increased number of osteoclasts. These changes have been reported in *Ggtl*^{-/-} mice as a causative change for dwarfism, cataracts, and osteopenia, respectively [2, 12]. In addition, reversal of these abnormalities by oral administration of NAC was shown in the *Ggtl*^{dwg/dwg} mice as in the *Ggtl*^{-/-} mice. Therefore, the histological characteristics and pathogenic mechanism of abnormalities of *Ggtl*^{dwg/dwg} and *Ggtl*^{-/-} mice are highly similar.

However, significant differences were observed in the progression of cataracts, mortality, and the change in the

number of osteoclasts in response to NAC. Cataract development in *Ggtl*^{dwg/dwg} mice begins around 3 weeks of age, while *Ggtl*^{-/-} mice already show more severe changes such as cortical vacuolization at this point. In addition, the alteration in the *Ggtl*^{dwg/dwg} mice was restricted to the cortical fibers of the lens, and cortical vacuolization was only observed at 9 weeks of age. Therefore, the progression and severity of diseases in *Ggtl*^{dwg/dwg} mice appear to be delayed and milder than those in *Ggtl*^{-/-} mice. The relationship between GSH levels in the lens and cataract development has been reported [15]. Since eye GSH levels in the *Ggtl*^{dwg/dwg} mice were higher than those in the *Ggtl*^{-/-} mice (15 vs. 5.5%, respectively, of eye GSH levels in the wild-type mice) [2], the difference in the progression and severity of cataracts may be caused by the difference in cellular GSH levels.

The difference in cellular GSH levels may also explain the differences in mortality. Studies using *Ggtl*^{-/-} mice showed that the depression of cellular GSH in the liver leads to DNA damage and impairment of cellular functions caused by mitochondrial dysfunction, which can result in cellular injury [17, 21]. Therefore, the difference in mortality may be attributed to the extent of cellular damage caused by the different levels of depression of intercellular GSH. Thus, owing to lesser depression of intercellular GSH, cellular damage induced by lower cellular GSH levels may be milder in *Ggtl*^{dwg/dwg} mice, resulting in no reduced life span in *Ggtl*^{dwg/dwg} mice unlike *Ggtl*^{-/-} and *Ggtl*^{enu1/enu1} mice. Therefore, an intracellular GSH level depending on residual GGT1 activity seems to be vital to the progression and severity of some types of abnormalities.

In human GGT deficiency, in addition to elevated plasma and urine GSH levels, specific symptoms in the central nervous system, including mental retardation, abnormal behavior, and absence seizure, have been observed [3, 8]. However, how GGT deficiency is pathologically related to these neurological symptoms remains unclear. The abnormal behaviors have not been reported in the *Ggtl*^{-/-} mice; however, severely abnormal behaviors such as circling and lurching have been observed in most of the *Ggtl*^{dwg/dwg} mice over 6 months of age (data not shown). The difference in neurological symptoms between *Ggtl*^{dwg/dwg} and *Ggtl*^{-/-} mice may be due to their life span, since about 85% of *Ggtl*^{-/-} mice do not survive past 6 months of age [14]. A certain survival time (≥ 6 months in the case of mice) with chronic depression

of cellular GSH may be required for the manifestation of these neurological abnormalities. To clarify the implication of GGT1 in the pathogenic mechanism of the abnormalities pertaining to the central nervous system, further investigations using an *in vivo* model that can be utilized for a longer period are necessary, and *Ggt1^{dwg/dwg}* mice will be an excellent model for such purpose. In conclusion, the *Ggt1^{dwg/dwg}* mouse is a useful animal model to elucidate not only the physiological roles of GGT1 in antioxidant systems through GSH homeostasis but also the pathogenesis of various diseases including neurological and age-related abnormality caused by impaired GGT1 function.

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