Liver-Specific γ-Glutamyl Carboxylase-Deficient Mice Display Bleeding Diathesis and Short Life Span

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Abstract

Vitamin K is a fat-soluble vitamin that plays important roles in blood coagulation and bone metabolism. One of its functions is as a co-factor for γ-glutamyl carboxylase (Ggcx). Conventional knockout of Ggcx causes death shortly after birth in homozygous mice. We created Ggcx-floxed mice by insertingloxPsequences at the sites flanking exon 6 of Ggcx. By mating these mice with albumin-Cre mice, we generated Ggcx-deficient mice specifically in hepatocytes (Ggcx⁺⁺/Liver/⁺⁺/Liver mice). In contrast to conventional Ggcx knockout mice, Ggcx⁺⁺/Liver/⁺⁺/Liver mice had very low activity of Ggcx in the liver and survived several weeks after birth. Furthermore, compared with heterozygous mice (Ggcx⁺⁺/Liver/+ or Ggcx⁺⁺/Liver/+), Ggcx⁺⁺/Liver/⁺⁺/Liver mice had shorter life spans. Ggcx⁺⁺/Liver/⁺⁺/Liver mice displayed bleeding diathesis, which was accompanied by decreased activity of coagulation factors II and IX. Ggcxfloxed mice can prove useful in examining Ggcx functions in vivo.

Introduction

Vitamin K is a fat-soluble vitamin, which is involved in blood coagulation and bone metabolism. One of the major functions of vitamin K is its role as a co-factor for γ-glutamyl carboxylase (Ggcx) [1]. Ggcx is responsible for the posttranslational modification of glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) by its carboxylase activity. Hitherto, 19 kinds of Gla proteins have been found, that is, coagulation factors II, VII, IX, and X; protein C, protein S, and protein Z [2]; osteocalcin [3]; nephrocalcin [4]; matrix Gla protein [5]; growth arrest specific-6 (Gas6) [6]; periostin [7]; Bgl-H3 [7]; proline-rich Gla protein 1 and 2 [8]; transmembrane Gla protein 3 and 4 [9]; upper zone of growth plate and cartilage matrix associated protein (UCMA; also called Gla-rich protein, GRP) [10]; and Ggcx itself, which was also shown to be γ-carboxylated [11]. Considering the various expression sites and functions of these Gla proteins, it is indicated that vitamin K is involved in many physiological and pathological processes by activating Ggcx.

On the other hand, we have previously demonstrated γ-carboxylation-independent vitamin K function, in which vitamin K is involved in the transcriptional regulation of nuclear receptor SXR/PXR [12]. We reported that SXR/PXR-dependent vitamin K functions are actually involved in the biological process in osteoblasts [13] and hepatocellular carcinoma cells [14].

To fully understand the function of vitamin K, it is vital to separate Ggcx-dependent and SXR/PXR-dependent vitamin K functions. Analysis of Ggcx knockout mice would be useful in examining Ggcx-dependent vitamin K functions in each tissue; however, this attempt has been hampered by the fact that Ggcx knockout mice die between embryonic day 9.5 and 18, and the few that survive to term die shortly after birth [15]. To overcome this limitation, we generated Ggcx-floxed mice that enabled organ-specific deficiency of Ggcx when bred with transgenic Cre mice that showed organ-specific expression of Cre recombinase. Here, we report a phenotype with liver-specific deficiency of Ggcx.
NotI. Genomic fragments were subcloned into pBluescript SK(+) 
The 5' homology arm of the construct was derived from an Asp718/HindIII genomic fragment containing intron 4, exon 5, and intron 5 of Ggcx. This fragment was subcloned into pBluescript SK(+) and then inserted into the 5' region of pNT1.1 between the NotI and SalI sites. The 3' homology arm of the construct was derived from a genomic fragment containing intron 6, exon 7, and intron 7 of Ggcx. This fragment was amplified with primers 5'-GGCTTAATATTAGGATATAGAAGACACC-3' and 5'-ATGGTACCTAGGAAAGCAGGAAGAAG-3' and inserted into the 3' region of pNT1.1 at the PacI and Asp718 sites. The genomic region containing exon 6 was amplified with primers 5'-AGGCTTACGGTGATTTCCGCCG-3' and 5'-ATGCAAAAAGCAAGGACCTG-3', and then inserted into pNT1.1 at a BamHI site between the 5'-loxP site and neomycin cassette. This resulted in a targeting vector with a neomycin cassette between exon 6 and 7 and a thymidine kinase cassette. This targeting construct was linearized with NotI and electroporated into D3 ES cells [16].

**Generation of Ggcx**\(^{\text{floxed/floxed}}\) **mice**

Colonies of ES cells carrying the recombinant allele were screened using 150 μg/ml of G418 and negatively selected using 2 μM gancyclovir. Selected cells were amplified and genomic DNA was screened by Southern blot analysis. The ES cell lines carrying the recombinant allele were subsequently used to generate chimeras by injection into 129/Sv blastocysts. The chimera mice were mated with wild type C57BL/6N mice. The F1 agouti offspring were analyzed for homologous recombination by Southern blotting and PCR analysis. The F1 offspring were intercrossed to generate Ggcx\(^{\text{floxed/floxed}}\) mice containing homozygous recombinant alleles.

**Generation of hepatocyte-specific Ggcx-deficient mice**

C57BL6/J mice containing transgenic constructs of mouse albumin enhancer/promoter and Cre recombinase modified to include a nuclear localization sequence (Alb-Cre) were purchased from the Jackson Laboratory. ROSA26-LacZ reporter mice were also obtained from the Jackson Laboratory. Hepatocyte-specific expression of Cre recombinase was confirmed by mating Alb-Cre mice with ROSA26-LacZ mice and assessing the β-galactosidase activity of the expressed LacZ gene, which is expected to be detected in cells expressing functional Cre recombinase. To generate hepatocyte-specific Ggcx-deficient mice (Ggcx\(^{\text{Alb-Cre/Alb-Cre}}\)), Alb-Cre mice were mated with Ggcx\(^{\text{floxed/floxed}}\) mice and F1 offspring were subsequently intercrossed.

**Southern blotting**

EcoRl digested genomic DNA—derived from ES cells or tail specimens—was electrophoresed through a 0.6% agarose gel, transferred to a Hybond N+ membrane (Amersham Bioscience), and hybridized with the 32P-labeled 164-bp sequence (ACACGTCTTTCTGATGGACATTTCCTCAGGAGAC- GGCGCCCTTACCTCGAGAGAATACCTTGATGGG- GCTGGATGTGGTCTCCTGAGATGCCCTTGCGCCACACTGCGACATGATGGCTATGTTCTGGG) in exon 3 of the Ggcx gene.

**Genotyping**

Genomic DNA derived from tail specimens was used as the template for PCR analysis. Tail cut was done before 3 weeks old or immediately after the mice died. The Cre recombinase gene was detected by amplifying a 654-bp fragment within the Cre gene with primers 5'-CCTGGAAAATGCTTCTGCGTTTGGC-3' and 5'-GAGGTAGATGCTGGTGCTGAGATG-3'. The 5'-loxP sequence was detected by amplifying the Ggcx sequence with primers 5'-ACCTAGGAGTTGTTCTTCATCT-3' and 5'-ATGCAATACCCAGGAGTTCTATGC-3' within intron 5, containing loxP and linker sequences, to yield a 454-bp fragment from the loxP-containing allele and 407-bp fragment from the wild type allele. Deletion of exon 6 in the liver was confirmed with primers 5'-CGGTACCTCACGCGGTTGTG-3' within exon 6 and 5'-TCTGATGCCGCTGACGGG-3' within intron 6. DNA samples derived from liver, spleen, kidney and heart of both Ggcx\(^{\text{Alb-Cre/Alb-Cre}}\) mice and control Ggcx\(^{\text{+/+}}\) mice. The DNA samples of same concentration (3 ng/μl) were used as templates for PCR analysis.

**Animal experiments**

Mice were housed in a temperature-controlled room (22°C) with a 12-h light/dark schedule, had free access to water, and were fed standard laboratory chow. When mice were sacrificed, anesthesia with an intraperitoneal injection of 2.5% avertin was employed to minimize suffering of animals. Exsanguination was done following anesthesia to ensure death.

**Ggcx activity assay**

FLEEL was purchased from Bachem (Philadelphia, PA). Lz-phosphatidylcholine (type VE) and CHAPS were obtained from Sigma Aldrich Japan (Tokyo, Japan). Vitamin K2 (menaquinone-4) was obtained from Eisai Co., Ltd. (Tokyo, Japan). The peptide ProFIX19, which contains the sequence AVFLDHNENKLNRPKRY, was synthesized by Genenet Co., Ltd. (Fukuoka, Japan). NaH14CO3 (specific activity, 58 mCi/mmol) was obtained from Amersham Biosciences Corp. (NJ).

Six-week old mice were anesthetized with an intraperitoneal injection of 2.5% avertin and the livers were excised for measurement of Ggcx activity. Mice were euthanized by exsanguination following liver excision. The Ggcx activity was measured as previously described [17]. The amount of 14CO2 incorporated into exogenous substrates was measured in reaction mixtures of 125 μl containing substrate (3.6 mM FLEEL), 222 μM reduced vitamin K (vitamin K7), 16 μM propeptide ProFIX19, 1.4 mM NaH14CO3 (5 μCi), 25 mM MOPS (pH 7.0), 500 mM NaCl, 0.16% (w/v) phosphatidylcholine, 0.16% (w/v) CHAPS, 8 mM DTT, and 0.8 M ammonium sulfate, unless stated otherwise. All of the assay components, except for the microsomal fraction, were prepared as master mixes. 14CO2 incorporation into peptide substrates (after an incubation period of over 30 min) was assayed using a scintillation counter. All assays were performed in triplicate.

**Coagulation factor activity assay**

Blood was collected from 6-week-old mice under anesthesia with an intraperitoneal injection of 2.5% avertin. Collected blood was immediately combined with one-tenth volume of 110 mM sodium citrate. Plasma was isolated by centrifugation for 15 min at 2500xg. The obtained plasma was analyzed with an automated blood coagulation analyzer (STA Compact, Roche, Basel, Switzerland) to determine factor II and IX activity using prothrombin (factor II) or factor IX-deficient plasma.
**Bleeding test**

Four-week-old mice were anesthetized with an intraperitoneal injection of 2.5% avertin. Their tails were cut to yield the same wound diameters. To evaluate bleeding time, filter paper was applied to the edge of the wound every minute, taking care not to dislodge the clot.

**Hematological examination**

Two ml of blood was collected from 6-week-old mice under anesthesia with an intraperitoneal injection of 2.5% avertin. Collected blood was mixed with anti-coagulants (1 mg of EDTA-2K and 20 μl of 3% EDTA-3K). The number of platelets was measured using the Advia 120 (Bayer, Dublin, Ireland).

**Life span analysis**

To evaluate lifespan, mice were kept with their littermates. Male and female mice were kept in separate cages without mating. They were kept until either natural death, or evidence of impending mortality necessitating euthanasia, such as unresponsiveness to touch, slow respiration, coldness to touch, a hunched up position with matted fur. Condition of the mice was monitored every two days.

**Statistical analysis**

Data are expressed as mean ± SEM. Differences between the mean values were analyzed using the unpaired Student’s t-test. Survival rates were plotted using the Kaplan-Meier method. Survival differences between the groups were analyzed using the log-rank test, for which p-values were adjusted by the Bonferroni method.

**Results**

**Generation of hepatocyte-specific Ggcx-deficient mice**

The mouse γ-glutamyl carboxylase (Ggcx) gene consists of 15 exons (Figure 1A). To disrupt the Ggcx gene, the targeting vector was designed to flank exon 6 with two loxP sequences, and a frameshift was generated by excision with Cre recombinase (Figure 1A). Insertion of loxP sequences by homologous recombination was confirmed with Southern blotting analysis (Figure 1B). To delete the Ggcx gene in the liver alone, albumin-Cre (Alb-Cre) transgenic mice were used. The cre recombinase gene is under the control of the albumin promoter, which is active only in hepatocytes from E16.5 embryos [18] and X are known to be vitamin K dependent. Therefore, we present study along with the clinical presentation of vitamin K deficiency indicate the relative importance of hepatic coagulation functions of vitamin K.

In the present study, we showed that liver-specific deficiency of Ggcx caused bleeding diathesis and short life span. We consider the massive bleeding in subcutaneous tissue or body cavity is a direct cause of death since we observed massive subcutaneous bleeding in most of the dead mice. It is also possible that local bleeding in vital organs such as brain can cause death due to bleeding diathesis.

**Discussion**

Mediation of post-transcriptional modification of substrate proteins by Ggcx is one of the major functions of vitamin K. So far, 19 proteins are known to be substrates of Ggcx and are expressed throughout body, indicating various physiological functions of vitamin K.

In the present study, we showed that liver-specific deficiency of Ggcx caused bleeding diathesis and short life span. We consider the massive bleeding in subcutaneous tissue or body cavity is a direct cause of death since we observed massive subcutaneous bleeding in most of the dead mice. It is also possible that local bleeding in vital organs such as brain can cause death due to bleeding diathesis.

Short life span of liver-specific Ggcx-deficient mice in the present study along with the clinical presentation of vitamin K deficiency indicate the relative importance of hepatic coagulation factors among Ggcx substrates. Coagulation factors II, VII, IX, and X are known to be vitamin K dependent. Therefore, we considered the decreased activity of these coagulation factors to be
designed to flank exon 6 of the Ggcx gene, and frame shift was generated by excision with Cre recombinase. Two loxP sequences (triangles) were inserted into introns 5 and 6. Neomycin cassette and EcoRI site were inserted into intron 6. B, Southern blot analysis of tail DNA. Homologous recombination allele generated a 7.4-kb fragment by EcoRI digestion. A representative figure is shown.

Both factor VII-deficient mice [22] and factor IX-deficient mice [23] displayed bleeding diathesis. The factor IX-deficient mice showed swollen extremities and extensive hemorrhagic lesions following mechanical trauma, although they survived for at least several weeks. In contrast, the factor VII-deficient mice survived to term and followed a normal Mendelian inheritance pattern. However, most of them died perinatally owing to intra-abdominal hemorrhage within 24 hours, and the remaining neonates died from intracranial hemorrhage in 24 days. Considering the aggressive bleeding of factor VII-deficient mice, the residual activity of Ggcx in Ggcx<sup>Alc5/Alc5</sup> mice may contribute to the survival. Furthermore, Ggcx activity before embryonic day 16.5 may have some preventive effect against postnatal bleeding.

In regard to the phenotypes of conditional deficiency of coagulation factors, factor VII-insufficient mice at the 0.7% expression level compared with wild-type mice could survive to adulthood despite displaying severely downregulated overall thrombin production and cardiac fibrosis at a young adult age [24]. Induction of prothrombin ablation in adulthood using Mx1-Cre caused fatal hemorrhagic events particularly in heart and brain [25]. Liver-specific Ggcx-deficient mice in the present study exhibit a longer life span in comparison with that of prothrombin-deficient mice. However, most of them died perinatally owing to intra-abdominal hemorrhage within 24 hours, and the remaining neonates died from intracranial hemorrhage in 24 days. Considering the aggressive bleeding of factor VII-deficient mice, the residual activity of Ggcx in Ggcx<sup>Alc5/Alc5</sup> mice may contribute to the survival. Furthermore, Ggcx activity before embryonic day 16.5 may have some preventive effect against postnatal bleeding.

Phenotype of Liver-Specific Ggcx-Deficient Mice

In regard to the phenotypes of conditional deficiency of coagulation factors, factor VII-insufficient mice at the 0.7% expression level compared with wild-type mice could survive to adulthood despite displaying severely downregulated overall thrombin production and cardiac fibrosis at a young adult age [24]. Induction of prothrombin ablation in adulthood using Mx1-Cre caused fatal hemorrhagic events particularly in heart and brain [25]. Liver-specific Ggcx-deficient mice in the present study exhibit a longer life span in comparison with that of prothrombin-deletion in adult mice, because the amount of coagulation factors in Ggcx-deficient mice are substantially decreased but even sufficient to survive for several weeks after birth. In comparison with factor VII-insufficient mice, however, it is assumed that severe insufficiency of multiple coagulation factors occurred in liver-specific Ggcx-deficient mice simultaneously.

It is intriguing that mice lacking fibrinogen, the final effector of the coagulation cascade, displayed similar phenotypes to those seen in Ggcx<sup>Alc5/Alc5</sup> mice [26]. They suffered from spontaneous abdominal hemorrhage, but long term survival was possible. In fibrinogen-deficient mice, pregnant female ones died from vaginal hemorrhage, which was also observed in Ggcx<sup>Alc5/Alc5</sup> mice.
In this study, we observed longer life spans of female Ggcx\textsuperscript{Dliver/Dliver} mice compared with male Ggcx\textsuperscript{Dliver/Dliver} mice. Notably, this sexual dimorphism of life span was also observed in fibrinogen-deficient mice [26], although the difference was smaller than that of Ggcx\textsuperscript{Dliver/Dliver} mice. Considering activities of Ggcx in the livers of Ggcx\textsuperscript{Dliver/Dliver} mice were not significantly different between male and female (Figure 2D), this sexual dimorphism may be owing to the difference in aggressiveness of behavior between males and females. Typically, males are more aggressive than females [27], which causing males more susceptible to injury. Another explanation for the sexual dimorphism of life span is the procoagulant activity of female sex hormone, estrogen. Clinically estrogen administration as oral contraceptives or hormone replacement therapy is known to be associated with higher risk of venous thrombosis [28]. In experiment using rats, estrogen was shown to prevent decline of prothrombin caused by vitamin K deficiency [29]. This report suggests higher concentration of estrogen in female mice may ameliorate bleeding diathesis due to Ggcx deficiency which is pathologically similar with vitamin K deficiency.

Interestingly, we observed higher activities of Ggcx as well as vitamin K-dependent coagulation factors in wild type male mice compared with female.
activity might be higher in one-month-aged male mice compared with one-month-aged females [31]. Since growth hormone has been known to contribute to sexual dimorphism in liver protein expression [32], it is assumed that the hormone will also exert difference in Ggcx activity.

In human, two missense mutations of GGCX gene were reported to cause hereditary bleeding disorder due to low activity of vitamin K-dependent coagulation factors [33,34]. In our study about 60% of female Ggcx<sup>−/−</sup> liver mice survived longer than 100 days indicated small portion of residual Ggcx activity detected in the liver of Ggcx<sup>−/−</sup> liver mice is sufficient to survive unless they got injured or pregnant. This is compatible with the clinical observation that patients with decreased carboxylase activity live several years before diagnosis [33,34].

In the current study, we successfully generated mice exhibiting liver-specific insufficiency of Ggcx activity. Because systemic Ggcx knockout mice do not live long after birth, our animal model enables to show the phenotype of liver-specific Ggcx deficiency for the first time and also will open up the possibility to evaluate in extra-hepatic organ-specific Ggcx activities using Cre recombinase driven by proper organ-specific promoters. Recently, several clinical and epidemiological studies have suggested extrahepatic actions of vitamin K. Its fracture-prevention effect has been proven in clinical studies [35,36], and vitamin K2 is used as a drug for osteoporosis treatment in several Asian countries. In epidemiological studies, low serum concentration of vitamin K was reported to be correlated with osteoarthritis [37], dementia [38], and coronary artery disease [39,40]. Moreover, a biosynthetic enzyme of menaquinone, which is an active form of vitamin K, was found to be expressed in extrahepatic organs [41]. These discoveries along with the many Ggcx substrates expressed throughout the body, suggest the extrahepatic function of Ggcx is worth investigating. The Ggcx-deficient mice created in this study would be useful in clarifying vitamin K action in the whole body.

**Acknowledgments**

We are grateful to A. Kawai, M. Tanaka, Y Esaki, and T. Tanaka for their technical assistance.
Author Contributions
Conceived and designed the experiments: KA TT KI SS KN MI. Analyzed the data: KA TT KI SS KN MI. Wrote the paper: KA TT KI KHI SI.

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