Requirement of Seminolipid in Spermatogenesis Revealed by UDP-galactose:Ceramide Galactosyltransferase-deficient Mice*

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Although seminolipid has long been suspected to play an essential role in spermatogenesis because of its uniquely abundant and temporally regulated expression in the spermatocytes, direct experimental evidence has been lacking. We have tested the hypothesis by examining the testis of the UDP-galactose:ceramide galactosyltransferase-deficient mouse, which is incapable of synthesizing seminolipid. Spermatogenesis in homozygous affected males is arrested at the late pachytene stage and the spermatogenic cells degenerate through the apoptotic process. This stage closely follows the phase of rapid seminolipid synthesis in the wild-type mouse. These observations not only provide the first experimental evidence that seminolipid is indeed essential for normal spermatogenesis but also support the broader concept that cell surface glycolipids are important in cellular differentiation and cell-to-cell interaction.

Seminolipid (3-sulfogalactosyl-1-alkyl-2-acyl-sn-glycerol) is the principal glycolipid in spermatozoa of mammals comprising, for example, approximately 3% of total lipids and more than 90% of total glycolipids in boar spermatozoa (1–3). During spermatogenesis, seminolipid is synthesized rapidly in the early phase of spermatocyte development and maintained in subsequent germ cell stages (4–6). This developmentally regulated rapid synthesis suggested a specific and possibly essential function of seminolipid in spermatogenesis (7) but experimental evidence has been lacking. Firm evidence in support of the speculation would have important bearing to the general concept that cell surface glycoconjugates are important in cellular differentiation, and cell-to-cell interaction (8).

Seminolipid is synthesized by sulfation of its precursor, galactosylglycerol (GaEAG)2. GaEAG is synthesized by UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.62), which, besides GaEAG, also synthesizes the major myelin galactolipid, galactosylceramide (GalCer), galactosylphosphoglycerine (psychosine), and galactosyldiacylglycerol (GalAA) (9, 10). The CGT-deficient mice recently generated by gene-targeting do not synthesize any of these products and subsequent derivatives of the products (11–14). Thus, the CGT-deficient mouse is an ideal experimental model to examine the consequences of lack of seminolipid to spermatogenesis. This report describes the first definitive evidence that deficient seminolipid biosynthesis indeed causes devastating disruption of the normal spermatogenic process.

EXPERIMENTAL PROCEDURES

Mice—The mice heterozygous for the disrupted Cgt gene (11) were originally supplied by Dr. B. Popko and maintained by backcrossing to C57BL/6N. Genotype was determined according to Coetzee et al. (11). WBB6F1 Rit/W-22001 and WBB6F1 Mgf/W-22001 mutant mice were purchased from Japan SLC, Inc., and C57BL/6N inbred mice were purchased from CLEA Japan, Inc.

Isolation of Testicular Germ Cells—Testicular germ cells were isolated from decapsulated testes of sexually mature male C57BL/6N mice (15).

RT-PCR Analysis—RNA was extracted using the RNeasy kit (Qiagen) or the TRIzol reagent (Life Technologies, Inc.). RNA samples were amplified using the Access RT-PCR system (Promega). For amplification of each transcript, the following primer sets were used: Cgt, 5′-tag-gaca-tcacagtta-gaagtctg-3′ and 5′-atgtgctga actaaggctgt-ga-3′; Dmc1, 5′-tag-tacctggagga-aagcagagcgtg-3′ and 5′-cttgctcgacatcaaggctgt-ga-3′; Sycp3, 5′-ggtggctga-agaatcagctgtgtg-3′ and 5′-cagctacaatttttcttcagc-3′; Hsp70-2, 5′-eagcagcaacgactac-3′ and 5′-ttcttgctctgctct-3′; Cign, 5′-atagtgctaa cccctggagac-3′ and 5′-gtagctgctgacacta-ac-3′; HoxA, 5′-tggcagctgctgaactc-3′ and 5′-ggtgagcagcagcactac-3′; Sprm-1, 5′-tcctgtgggctgccac-3′ and 5′-ccceagaggcttgcta-3′; Cnca1, 5′-catgtgctgca gaaacagagcagc-3′ and 5′-ccctagtgtctggagacatcttc-3′; Cremt, 5′-ggagcagcaagagacatc-3′ and 5′-cagctacgtagctacagtag-3′; Hsc70t, 5′-tcctggcagctagcaggc-3′ and 5′-agctctctctctctgcttct-3′; Hprt, 5′-ctctggatcatagccactagc-3′ and 5′-gtaaggggcttctacacac-3′.

Histological Analysis—Testes were dissected and fixed in Bouin’s solution overnight. After dehydroxylation, tissues were embedded in paraffin and 6-μm sections were stained for the periodic acid-Schiff (PAS) reaction followed by hematoxylin staining. In situ labeling of apoptotic cells was performed on the tissue sections prepared in the same way using the MEBSTAIN Apoptosis kit II (Medical & Biological Laboratories Co.), according to the manufacturer’s protocol except that the Vectastain elite ABC kit (Vector) was used for the detection system.

Lipid Extraction and Analysis—The total lipid extract (16) was analyzed by two-dimensional TLC using the solvent systems, chloroform/methanol/water (60:35:8, by volume) containing 0.2% CaCl2 (first direction) and chloroform/methanol/acetic acid/water (8:2:4:2, by volume) (second direction). To differentiate GalCer and GlcCer, the solvent for second direction was replaced by chloroform/methanol/water (60:35:8, by volume) containing 0.2% CaCl2 (first direction), according to the manufacturer’s protocol except that the Vectastain elite ABC kit (Vector) was used for the detection system.

1 The abbreviations used are: GaEAG, galactosyl-1-alkyl-2-acetylglycerol; CGT, UDP-galactose:ceramide galactosyltransferase; GalAA, galactosyldiacylglycerol; GalCer, galactosylceramide; LSIMS, liquid secondary ion mass spectrometry; RT-PCR, reverse transcriptase-mediated polymerase chain reaction; TLC, thin-layer chromatography; bp, base pairs.

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RESULTS

Anatomical Defects in Reproductive Organs of CGT-deficient Male Mice—Homozygous affected male mice develop severe clinical phenotype early, and thus, it was impossible to ascertain clinically if they were fertile. Anatomically, however, the testis was dramatically impaired in \( \text{Cgt}^{-/-} \) male mice. The average testis weight of \( \text{Cgt}^{-/-} \) mice (24.9 ± 1.7 mg; \( n = 8 \)) was only one-fourth of that of wild-type littermates (97.3 ± 3.5 mg; \( n = 6 \)) at 8 weeks of age. The size of the epididymis was also reduced in the \( \text{Cgt}^{-/-} \) male mice. The seminal vesicle, and also the kidney, were smaller but only in proportion to the smaller body of the affected mice (70% of control littermates).

Expression of Cgt mRNA in Testicular Germ Cells—RT-PCR analysis of Cgt transcripts in normal adult mice showed that this message was expressed in testis in addition to brain and kidney (Fig. 1a). Transcripts of the Cgt gene were expressed in the testis from normal mice of all ages and in testicular germ cells of adult mice but were extremely reduced in the testes of \( \text{Kit}^{W/W} \), \( \text{MgfSl}^{Sl/d} \), and \( \text{jsd}^{/jsd} \) mutants (Fig. 1b), in which only undifferentiated spermatogonia and somatic cells were found in the seminiferous tubules (20, 21). The result with the \( \text{Kit}^{W/W} \) mouse is consistent with the deficiency of seminolipid in this mutant mouse (5). Seminolipid levels in other mutants are not known. These results indicated that Cgt mRNA is expressed only in germ cells at the stage later than spermatogonia but not in the somatic cells of the testis. Thus, loss of CGT enzyme activity could cause functional defects specifically in the testicular germ cells.

Disruption of Spermatogenesis in CGT-deficient Mice—Histological examination showed that \( \text{Cgt}^{-/-} \) males had a complete disruption of spermatogenesis (Fig. 2, a and b). Testicular germ cells after meiosis were absent in the seminiferous tubules of \( \text{Cgt}^{-/-} \) mice, whereas spermatogonia and early spermatocytes appeared normal. The Leydig cells and Sertoli cells also appeared normal in \( \text{Cgt}^{-/-} \) mice. The abnormal seminiferous tubules in adult \( \text{Cgt}^{-/-} \) mice could be divided roughly to three stages of development. The tubules of the first stage had a simple structure consisting of a layer of spermatogonia (Fig. 2c). The second stage consisted of multilayers of spermatocytes at the pachytene stage, together with spermatogonia (Fig. 2d). No spermatocytes beyond this stage could be found in these tubules. The third stage of tubules exhibited cellular degeneration and formation of syncytial multinucleated cells, which were probably produced from spermatocytes at the late pachytene stage (Fig. 2e). The TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay indicated that the degenerating cells were undergoing apoptotic cell death (Fig. 2f).

To verify the cellular identities and developmental stages disrupted in germ cells in the \( \text{Cgt}^{-/-} \) testis, testes of \( \text{Cgt}^{-/-} \) juvenile mice were histologically compared with those of normal littermates at each stage. The first wave of spermatogenesis in juvenile mice results in the appearance of spermatocytes by 10 days after birth (22). At 14 days, spermatocytes at the pachytene stage emerge. Until 15 days, morphological features of the testis of the \( \text{Cgt}^{-/-} \) mice were indistinguishable from those of wild-type and the \( \text{Cgt}^{+/+} \) mice described above were readily detectable in the testis of the \( \text{Cgt}^{-/-} \) mice (Fig. 2, i and j). These results indicated that morphological defects developed during the late pachytene stage, but not in the zygote and early pachytene stages, of the spermatocyte development.

Arrest of Genetic Program of Spermatogenesis in CGT-deficient Mice—We further tried to verify the stage of the developmental arrest of spermatogenic cells in \( \text{Cgt}^{-/-} \) mice using RT-PCR analyses for transcripts of genes, expression of which is known to be developmentally programmed during spermatogenesis (Fig. 3). Expression of \( \text{Dmc1} \) gene occurs in early stages of spermatogenesis in wild-type mice (23) and that of \( \text{Sycp3} \) in early meiosis (24). The transcripts from these genes were present in testis of \( \text{Cgt}^{-/-} \) mice. Expression of \( \text{Hsp70-2} \), \( \text{Clgn} \) (encoding calmodin), \( \text{HoxA4} \), \( \text{H1f3} \) (encoding histone H1t), \( \text{Sprrm-1} \), and \( \text{Cre} \) genes was reduced in \( \text{Cgt}^{-/-} \) mice. These genes are reported to be expressed in primary spermatocytes at the late pachytene stage (25–30). Expression of \( \text{Ccnal} \) (encoding cyclin A1) gene is reported to occur at the end of prophase of meiosis I (31). Expression of this gene was drastically reduced in \( \text{Cgt}^{-/-} \) mice. Expression of the \( \text{Hsc70t} \) gene begins in spermatids in wild-type mice (15). Transcripts from this gene were undetectable in \( \text{Cgt}^{-/-} \) mice. These results collectively indicated that spermatogenic cells of \( \text{Cgt}^{-/-} \) mice arrested their differentiation program before reaching the first meiotic division. They are consistent with the morphological observation that differentiation of the spermatogenic cells of \( \text{Cgt}^{-/-} \) mice is affected at the late pachytene spermatocyte stage.

Lack of Seminolipid and Its Precursor in Testes of CGT-deficient Mice—By LSIMS, the putative bands on the TLC plates were identified as seminolipid (32) and GalEAG with the major molecular species of 16:0 alcohol and fatty acid (data not shown). At 10 days after birth, normal testes contained definite levels of GalEAG (55 nmol/g wet tissue) and seminolipid (77 nmol/g) (Fig. 4a). By 12 days just before spermatocytes normally begin to appear at the zygote stage (22), the levels of seminolipid and GalEAG dramatically increased to 483 and 214 nmol/g, respectively. The level of seminolipid then increased gradually up to 699 nmol/g at 17 days after birth, while that of GalEAG remained constant (208 nmol/g at 17 days) (Fig. 4, b–d). The appearance and increase of seminolipid at 10 and 12 days, respectively, coincide with the beginning of incorporation of \( ^{35} \text{S} \)-sulfate into seminolipid of mouse testis at
11 days (2). At 7–12 weeks, the seminolipid level was reduced in Cgt<sup>−/−</sup> mice (557 nmol/g) compared with that in wild-type mice (816 nmol/g). Both in wild-type mice and heterozygotes, the adult testis had reduced level of GalEAG (−70 nmol/g) as compared with the juvenile testis of 12–17 days of age (−200 nmol/g, see above) in agreement with the levels reported in other mammalian species (3). In contrast, seminolipid and GalEAG were not detectable in the testis of the Cgt-deficient mouse at any stage of development (Fig. 4, e and f).

Among monohexosylceramide (HexCer), only glucosylceramide (GlcCer), but not GalCer, was detected in all genotypes (Fig. 4). In the testes of wild-type mice and heterozygotes, the major molecular species of GlcCer contained 16:0 fatty acid and d18:1 sphingosine, while appearance of a 2-hydroxy 16:0-containing molecular species of GlcCer contained 16:0 fatty acid and d18:1 ide (GlcCer), but not GalCer, was detected in all genotypes (Fig. 3).

DISCUSSION

Seminolipid, as its name implies, is present at an unusually high concentration in normal testis and its appearance is developmentally regulated (for reviews, see Refs. 2 and 3). This led to a long standing speculation that seminolipid plays an important role in the normal spermatogenesis process in the testis. However, evidence was only circumstantial and no direct experimental proof for the hypothesis has been available.

Our observations have clearly established that 1) mice genetically deficient in Cgt are unable to synthesize the precursor, GalEAG, and consequently its sulfated derivative, seminolipid itself; 2) the spermatogenic cells are the only cells in normal testis that express Cgt transcripts and thus are able to synthesize seminolipid; 3) seminolipid and its precursor become readily detectable in normal testis at 12-day-old mice in which spermatocytes develop into the zygote stage, prior to the pachytene stage; 4) the size of the testis of Cgt-deficient mice is one-fourth of normal at 8 weeks; 5) the cycle of spermatogenesis is terminated in Cgt<sup>−/−</sup> male mice at the late pachytene stage, when the spermatogenic cells that appear to develop and differentiate normally up to this stage degenerate and disappear; and 6) apoptotic cell death may well be the mechanism underlying their degeneration. Collectively, these data indicate strongly that Cgt is required for transition of primary spermatocytes through the late meiotic stages and that this process is mediated by seminolipid, thus providing the first experimental evidence for the long standing conjecture that seminolipid is essential for normal spermatogenesis.

While our results establish that seminolipid in normal spermatogenesis is essential, the precise molecular mechanisms of two processes remain to be fully understood: the mechanism of the male germ cell degeneration in the absence of seminolipid and the mechanism of the seminolipid function in normal spermatogenesis. Our morphological observation suggests that apoptotic cell death is the underlying mechanism in the degeneration of primary spermatocytes at the late pachytene stage of Cgt<sup>−/−</sup> mice. Generally, germ cells lacking a gene that is essential for normal spermatogenesis degenerate by apoptosis after the arresting step (for a review, see Ref. 33). The Cgt gene can now be recognized as one of the essential genes for normal spermatogenesis, and apoptosis in these cells could be a consequence of Cgt deficiency.

What triggers the apoptotic process in the germ cells of Cgt<sup>−/−</sup> male mice can only be speculated. There is evidence...
that cellular concentration of certain lipids may be a factor to initiate apoptosis. CGT-deficient mice do not synthesize GalCer and galactosylsulfatide in the brain, but the presence of 2-hydroxy fatty acid-containing GlcCer may partially compensate for the absence of GalCer and galactosylsulfatide (11). In the kidney of CGT-deficient mice, GalCer and galactosylsulfatide are also absent but here again a partial compensation by more polar sulfoglycolipids occurs.3 In the testis of the CGT-deficient mice, however, no lipids increase to compensate for the loss of seminolipid and GalEAG (11). The lack of seminolipid and GaIEAG without compensatory increases in other lipids may be the primary factor responsible for apoptosis of the germ cells in Cgt−/− male mice.

The function of seminolipid in the normal spermatogenesis is even less clear. Several lines of evidence suggest that the temporal and spatial coordination of germ cell differentiation may be mediated by surface interactions between germ cells and Sertoli cells (for a review, see Ref. 34). Immunological techniques have demonstrated that seminolipid is present on the surface of primary spermatocytes and round spermatids but not on the spermatogonia in rat (35). Our present study supports the general idea that cell surface glycolipids are functionally important in germ cell differentiation and/or interactions with other cell types.

It must be pointed out that our study leaves one minor ambiguity. Since the CGT-deficient mouse generates neither seminolipid nor its precursor, GalEAG, precise dissection of the functions of the precursor and its sulfated end product, seminolipid, is difficult. The gene of 3′-phosphadenthylsulfate:galactosylceramidase 3′-sulfotransferase (EC 2.8.2.11) that sulfates GalEAG to seminolipid, as well as GalCer to galactosylsulfatide, has recently been cloned (36, 37). The anticipated sulfotransferase knockout mouse should be able to provide the definitive answer as to whether both GalEAG and seminolipid or only either GalEAG or seminolipid are essential for normal spermatogenesis.

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