Oocyte Galα1,3Gal Epitopes Implicated in Sperm Adhesion to the Zona Pellucida Glycoprotein ZP3 Are Not Required for Fertilization in the Mouse*

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The Galα1→3Gal structure is displayed on the zona pellucida glycoprotein ZP3 on murine oocytes. This trisaccharide has been implicated in sperm-zona pellucida adhesive events thought to be essential to fertilization in the mouse. To determine directly if this molecule is required for fertilization, we have generated mice that are deficient in a gene (α1,3GT) encoding the UDP-Gal:β-o-Gal-α1→3 Gal-galactosyltransferase enzyme responsible for Galα1→3Gal synthesis and expression. These mice develop normally and exhibit no gross phenotypic abnormalities. The Galα1→3Gal epitope is absent from the vascular endothelium and other tissues in α1,3GT (−/−) adult mice. By contrast, α1,3GT (−/+) mice, like humans, develop naturally occurring anti-α-galactoside antibodies normally absent in wild type mice. Female α1,3GT (−/−) mice yield oocytes that are devoid of the Galα1→3Gal epitope; however, these mice are fully fertile. These observations indicate that the Galα1→3Gal moiety is not essential to sperm-oocyte interactions leading to fertilization or to essentially normal development. They further suggest that α1,3GT (−/−) mice will find utility for exploring approaches to diminish anti-Gal-dependent hyperacute xenograft rejection, which presents a major barrier to the use of porcine and other non-primate mammalian organs for xenotransplantation in humans.

Fertilization in mammals involves an adhesive interaction between sperm and the zona pellucida, a glycoprotein-containing shell that surrounds the oocyte. Sperm receptor activity of the murine oocyte resides in the zona pellucida glycoprotein ZP3 (1). Sperm recognition of murine ZP3 depends upon O-linked oligosaccharides displayed by ZP3 (Ref. 2; reviewed in Refs. 3–5). Treatment of purified egg ZP3 and ZP3-derived O-linked oligosaccharides with α-galactosidase eliminates sperm receptor activity (6). These observations have been taken to imply that terminal α-galactosides on ZP3 glycoconjugates are critical for sperm binding activity (6). This notion is supported by more recent observations demonstrating that structurally defined bi- and tetraantennary blood group I-related oligosaccharides containing terminal Galα1→3Gal moieties inhibit binding of sperm to eggs in a dose-dependent manner (7).

In the mouse, at least one UDP-Gal:β-o-Gal-α1→3Gal-galactosyltransferase (α1,3GT) is responsible for the synthesis of terminal Galα1→3Galβ1→4GlcNAc trisaccharides from common lactosamine-terminated glycoconjugates (8, 9). Mice and other placental mammals express the Galα1→3Galβ1→4GlcNAc trisaccharide products of α1,3GT on a variety of glycoproteins and in a variety of tissues (10, 11). Aside from the postulated role of Galα1→3Gal moiety in murine fertilization, the function(s) of this structure are not known.

By contrast, humans, apes, and Old World monkeys lack the ability to synthesize these oligosaccharide moieties, because the genetic homologues of the murine α1,3GT locus are pseudogenes incapable of encoding a functional α1,3GT (12, 13). Consequently, these latter species are reciprocally replete with immunoglobulins of all classes directed against terminal Galα1→3Gal epitopes (14, 15). These antibodies are presumed to arise through immunization by environmental antigens similar or identical to the Galα1→3Gal epitope (16). In humans these natural antibodies (termed anti-Gal) comprise approximately 1% of circulating IgG, as well as a significant fraction of circulating IgM class antibodies (14, 17). Anti-Gal antibodies are clinically important in the context of the proposed use of porcine and other non-primate mammalian organs to circumvent the shortage of human organs for transplantation purposes (reviewed in Refs. 18 and 19). Anti-Gal antibodies serve to initiate hyperacute rejection of xenografts derived from such mammalian species, via complement-mediated cytolytic events involving terminal Galα1→3Galβ1→4GlcNAc glycoconjugates expressed by the vascular endothelium of the xenograft (20, 21).

To directly address the role of Galα1→3Gal containing oligosaccharides in fertilization in the mouse, we have used a gene disruption approach in embryonic stem cells (22) to generate mice homozygous for a null α1,3GT allele. α1,3GT (−/−) mice are deficient in the expression of Galα1→3Gal epitopes on oocytes but are as fertile as their wild type litter mates, indicating that Galα1→3Gal epitopes are not essential to sperm-oocyte binding in this species. As with humans, apes, and Old World monkeys, α1,3GT (−/−) mice maintain naturally occurring anti-Gal antibodies but are deficient in the expression of Galα1→3Gal epitopes on vascular endothelium and other tissues. These observations imply that the inactivated α1,3GT gene represents the only functional murine α1,3GT locus, and they suggest that the α1,3GT (−/−) mouse may prove useful as a small animal model for studying approaches that can diminish anti-Gal-dependent hyperacute organ transplant rejection.

**EXPERIMENTAL PROCEDURES**

Generation of α1,3GT (−/−) Mice—A genomic clone of the α1,3GT locus was isolated from the 129/SV mouse strain and restricted with NdeI-MluI, and the resulting 12-kilobase fragment was cloned into pGem-5 (Stratagene). A neomycin resistance cassette, pgkNeo, was inserted into the SalI site within the catalytic domain, and a 500-base pair BstEII-NotI fragment was subsequently removed from a position

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†The abbreviations used are: α1,3GT, UDP-Gal:β-o-Gal-α1→3Gal-galactosyltransferase; ES, embryonic stem cell(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA/B4, B. simplicifolia isolectin B4.
~900 base pairs 3’ of the pgkNeo insertion. A thymidine kinase cassette (pgkTK) (23) was then inserted into the targeting vector at a position corresponding to the BstEII site. The Neo insertion disrupts the largest coding exon in the α1,3GT gene (8); fusion of pgkNeo sequences to this exon results in premature termination codons in all three exonic reading frames. D3 embryonic stem cells (ES) (24) were electroporated and selected by standard methods (25). Homologous recombination of the targeting vector with the native allele was detected in individual ES clones by a nested polymerase chain reaction strategy (Fig. 1a, solid arrowheads). Polymerase chain reaction-positive ES clones were expanded and were subjected to extensive restriction analysis by Southern blotting of genomic DNA. ES clones containing a single, homologously integrated targeted allele were used to generate chimeric mice via blastocyst injection, as described previously (26). Progeny from two ES lines (1G7 and 1F3) were used for subsequent experiments.

Determination of Mouse Fertility—Chimeric males resulting from ES cell injections were mated with F1(5C5BL/6j × DBA/2j) females, to yield heterozygous (α1,3GT (+/−)) progeny. The F1(129SV × 5C5BL/6j) × DBA/2j × α1,3GT (+/−) littermates were bred to yield F2 α1,3GT (+/−), (+/−), and (−/−) progeny. F2 α1,3GT (−/−) females were housed with proven fertile wild type males and control wild type females. F2 α1,3GT (−/−) males were housed with F2 α1,3GT (−/−) female litter mates and wild type control females. Litter sizes were determined based on the number of pups found on the day of birth.

Immunological Assays—Lungs were removed from α1,3GT (+/+), α1,3GT (−/−) and α1,3GT (+/−) littermates, fixed in 4% paraformaldehyde, and embedded in paraffin, and 4 μm sections were made. Sections were preincubated with 50 μg/ml green coffee bean α-galactosidase (Sigma) or with buffer alone. Endogenous peroxidase was quenched with 0.3% H2O2 in methanol. Sections were preblocked with 1% crystalline BSA (Sigma) in PBS, and were then incubated for 2 h at room temperature with 20 μg/ml biotinylated anti-α-galactosidase (Vector), followed by peroxidase ABC staining (Vector). Rabbit erythrocyte agglutination assays were performed as described previously (14), with the exception that sera were also preincubated with 10 μg/ml of either Synsorb H (Fucα1–2Galβ1–4GlcNAc) or Synsorb 115 (Galβ1–3Gala1–4GlcNAc) beads (Chembiomed, Edmonton, Alberta, Canada).

Detection of α Galactosidases on Oocytes—Four-week-old female wild type and α1,3GT (−/−) littermates produced by F1 heterozygous crosses were superovulated, and oocytes were isolated from the ovarides in M2 medium supplemented with 300 μg/ml hyaluronidase (Sigma). Oocytes were transferred through serial changes of buffer containing BSA, 0.4% BSA, and were then incubated for 1 h at 37°C in buffer containing 1:100 biotinylated anti-human IgG and IgM (Vector), followed by peroxidase ABC staining (Vector). Rabbit erythrocyte agglutination assays were performed as described previously (14), with the exception that sera were also preincubated with 10 μg/ml of either Synsorb H (Fucα1–2Galβ1–4GlcNAc) or Synsorb 115 (Galβ1–3Gala1–4GlcNAc) beads (Chembiomed, Edmonton, Alberta, Canada).

RESULTS AND DISCUSSION

A targeted disruption of the murine UDP-Gal:αβ-Galα1,3Galα1,3Galα1→3Gal glycosyltransferase (α1,3GT) gene in embryonic stem cells (ES) was completed as shown in Fig. 1a. F1 heterozygous (α1,3GT (+/−)) littermates were intercrossed to yield viable progeny with genotype frequencies (22%/−, 50%/−, and 28%/−) corresponding to a Mendelian inheritance pattern, indicating that homogygosity for the null α1,3GT allele is compatible with essentially normal intrauterine development. Mice that are homozygous for the null allele do not differ in size or appearance from their wild type litter mates. The major organs of the α1,3GT (−/−) animals are grossly and histologically normal, as are the levels of a variety of serum analytes. Total and differential blood leukocyte counts, red cell counts, and platelet counts are not significantly different between the α1,3GT (−/−) mice and wild type control mice.

We used human Galα1,3Gal α1,3Gal antibodies to confirm that the α1,3GT (−/−) mice are deficient in Galα1,3Gal expression. In humans, at least 1% of the circulating IgG class antibodies, and substantial amounts of circulating IgM class antibodies, are directed against terminal Galα1,3Galβ1–4GlcNAc-containing oligosaccharides (14, 17). By contrast, human tissues are essentially devoid of Galα1,3Gal epitopes, because a functional α1,3GT locus is apparently not present in humans (12, 13). In humans, “naturally occurring” polyclonal antibodies directed against the Galα1,3Gal epitope (27) (termed “anti-Gal” antibodies) are presumed to occur as a consequence of continuous immunization by gastrointestinal flora containing glycoconjugates with terminal α−galactosides (16). As noted previously (10), human anti-Gal antibody detects α−galactosidase-susceptible Galα1,3Gal epitopes on a variety of wild type murine cells, including vascular endothelium (Fig. 2, a and b). By contrast, the vascular endothelium of α1,3GT (−/−) mice is devoid of detectable terminal α−galactosides (Fig. 2c). α1,3GT activity, normally present in murine spleen cells, is not detectable in α1,3GT (−/−) splenocytes (data not shown).

The reciprocal relationship between the absence of Galα1,3Gal epitopes and the presence of anti-Gal antibody observed in humans and some other primates (12) is recapitulated in the α1,3GT (−/−) mice. Sera from α1,3GT (−/−) mice directly agglutinate Galα1,3Gal-positive (14, 28) rabbit erythrocytes, whereas sera from α1,3GT (+/+) mice, as expected, do not and thus are devoid of anti-Gal antibody activity. The rabbit erythrocyte hemagglutinating activity present in α1,3GT (−/−) sera can be removed by preincubation of the sera with immobilized synthetic Galα1,3Galβ1–4GlcNAc structures, whereas removal of hemagglutinating activity does not
occur when the sera are preabsorbed with Fucα1→2Galβ1→4GlcNAc structures (Fig. 2d). Sera prepared from α1,3GT (−/−) mice contain antibodies that also bind to murine laminin, a glycoprotein containing terminal α-galactosides (29), but do not bind α-galactosidase-treated laminin. By contrast, sera from α1,3GT (+/+) mice do not bind to murine laminin (data not shown). These observations indicate that the α1,3GT (−/−) mice maintain naturally occurring anti-Gal antibodies and indicate that these mice are therefore essentially deficient in the expression of terminal Galα1→3Gal moieties.

Studies in vitro indicate that terminal α-galactosides displayed by O-linked glycans on the mouse zona pellucida glycoprotein ZP3 are required for the binding of sperm to the oocyte (2–4). These glycoconjugates are easily demonstrated on the zona pellucida of wild type oocytes (Fig. 3b), using a lectin (BSIB4) that specifically recognizes these molecules (30). By contrast, oocytes obtained from α1,3GT (−/−) females do not stain with this lectin (Fig. 3e). The same result was also observed by staining oocytes with human anti-Gal (data not shown). The loss of the ability to detect oocyte α-galactosides is not due to a blocking effect of maternal anti-Gal immunoglobulins bound to the oocyte, since anti-mouse immunoglobulins did not interact with these oocytes (data not shown). These observations directly demonstrate that the α1,3GT locus determines oocyte expression of terminal α-galactosides.

Table I summarizes breeding studies completed to determine if fertility is affected by absence of zona pellucida terminal α-galactosides consequent to nullizygosity at the α1,3GT locus. In matings between α1,3GT (−/−) females and fertile wild type males of the same genetic background, we observed fertility rates and litter sizes equivalent to those observed in control matings involving α1,3GT (+/−) and α1,3GT (+/+). These observations demonstrate that absence of zona pellucida terminal α-galactosides is compatible with normal fecundity and indicate that terminal α-galactosides do not represent an essential component of the mouse oocyte sperm receptor(s). This conclusion leaves open the possibility that Galα1→3Gal-containing blood group I-related oligosaccharides capable of blocking sperm-egg binding (7) are instead responsible for sperm-egg adhesion during fertilization. Absence of an essential role for terminal Galα1→3Gal structure in fertilization is also consistent with an alternative hypothesis that murine sperm-egg adhesion during fertilization is accomplished through an interaction between terminal N-acetylgalactosamime moieties on the oocyte and surface-localized β(1,4)galactosyltransferase on murine spermatids (31).

In humans, naturally occurring anti-Gal antibodies of the type found in the α1,3GT (−/−) mice present a major obstacle to the use of porcine and other non-primate organs for human xenotransplantation. These antibodies bind to terminal α-galactosides on vascular endothelial cells of these mammals (17, 18) and mediate hyperacute xenograft rejection (19, 21) through complement-dependent endothelial cell cytotoxicity. Early attempts to completely block these interactions in vivo met with limited success (21). More recent work involving transgene-directed overexpression of complement inhibitors in the xenograft has shown substantial promise as a means to mitigate anti-Gal-dependent hyperacute xenograft rejection (32). Currently, Old World monkeys, which are naturally deficient in the Galα1→3Gal epitope but reciprocally replete with
animals can represent a substantial impediment to experimental considerations associated with the care of these large experimental animal recipients for such studies; cost and logistical considerations associated with the care of these large animals can represent a substantial impediment to experimental progress in this area. The α1,3GalT (−/−) mice we describe here may represent a useful alternative small animal for this work, since it can be anticipated that the naturally occurring anti-Gal antibodies in an α1,3GalT (−/−) murine graft recipient will lead to hyperacute graft rejection of a transplanted organ taken from an α1,3GalT (+/+) Galα1−3Gal-positive, but otherwise syngeneic donor mouse. The extensive experience with organ transplants in mice (33), the well-defined histocompatibility locus in this species (34), and highly developed systems for murine transgenesis represent additional advantages of this system for the study of anti-Gal-dependent hyperacute organ transplant rejection. Studies are currently in progress to study hyperacute transplant rejection utilizing these α1,3GalT (−/−) mice.

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Table I

| Mating                        | Average litter size |
|-------------------------------|---------------------|
| Maternal transmission         | 7 ± 2 (24)          |
| (−/−) × (+/+), paternal       | 5 ± 2 (48)          |
| transmission                  |                     |
| Heterozygous                  | 7 ± 1 (160)         |
| Cross                         |                     |
| Null cross                    | 5 ± 2 (47)          |