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Genetics

A novel functional role for apolipoprotein B in male infertility in heterozygous apolipoprotein B knockout mice

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ABSTRACT **Male infertility, affecting as many as 10% of the adult population, is an extremely prevalent disorder. In most cases, the cause of the condition is unknown, and genetic factors that might affect male fertility, other than some sequences on the Y chromosome, have not been identified. We report here that male mice heterozygous for a targeted mutation of the apolipoprotein B (apo B) gene exhibit severely compromised fertility. Sperm from these mice failed to fertilize eggs both *in vivo* and *in vitro*. However, these sperm were able to fertilize eggs once the zona pellucida was removed but displayed persistent abnormal binding to the egg after fertilization. *In vitro* fertilization-related and other experiments revealed reduced sperm motility, survival time, and sperm count also contributed to the infertility phenotype. Recognition of the infertility phenotype led to the identification of apo B mRNA in the testes and epididymides of normal mice, and these transcripts were substantially reduced in the affected animals. Moreover, when the genomic sequence encoding human apo B was introduced into these animals, normal fertility was restored. These findings suggest that this genetic locus may have an important impact on male fertility and identify a previously unrecognized function for apo B.**

Apolipoprotein B (apo B) is one of several apolipoproteins that play key roles in lipoprotein metabolism (1). Plasma levels of apo B are an important risk factor for coronary heart disease, and apo B is the ligand for receptor-mediated removal of low density lipoprotein particles from circulation (1, 2). Mutations in apo B result in familial hypobetalipoproteinemia in humans (1, 3). These data and studies of mutations in the other apolipoprotein genes have established a central role for these molecules in determining the risk of cardiovascular disease.

Recent studies, however, have suggested involvement of one or more apolipoproteins in other organ systems as well. For example, a variant of apolipoprotein E, apolipoprotein E4, correlates strongly with development of Alzheimer disease (4). While the mechanism by which inheritance of this genetic variant predisposes to Alzheimer disease is unknown, the findings provide impetus for examining possible roles of the apolipoproteins outside the cardiovascular system, with the ultimate goal of better understanding, predicting, and treating a wider variety of human diseases.

In an effort to produce animal models of apo B deficiency, we and others have inactivated the mouse gene that encodes this protein by use of targeted mutagenesis in embryonic stem cells (5, 6). Homozygotes for null apo B alleles developed neural tube defects and died *in utero* (5, 6), while homozygotes for a truncated apo B allele showed incomplete penetrance for embryonic lethality (7). Heterozygotes for the null allele showed reduced cholesterol levels (5, 6) and we also noticed infertility in a majority of the male heterozygotes that were derived from female heterozygzgotes of the F1 generation (5). These latter findings led us to explore a possible role for apo B in male fertility. Affecting as many as 10% of men, infertility is one of the most common disorders in the United States (8). However, despite the prevalence of this problem, no genetic polymorphisms other than several mutations on the Y chromosome (9–11) have been correlated with male infertility. We report here that male mice heterozygous for a null apo B allele (i.e., apo B +/− mice) have reduced fertility which is associated with a variety of sperm abnormalities frequently seen in infertile men.

MATERIALS AND METHODS

Mice. Mice containing one null apo B allele (apo B +/−) were generated by gene targeting techniques in embryonic stem cells as described (5). All apo B +/− mice used in these studies were originally derived from female apo B +/− mice of the F1 generation (5), and they were offspring from the first generation of backcross (N2) between female F1 apo B +/− mice and wild-type male C57BL/6J mice. These mice were of mixed genetic background and contained 75% and 25% of genetic material from the two parental strains, C57BL/6J and SV129, respectively. Mice of C57BL/6J, B6CBAF$_1$/J and B6D2F$_1$/J strains were purchased from The Jackson Laboratory.

Human apo B transgenic (HuBTg) mice were generated as described (12). Female apo B +/− mice were bred with male HuBTg mice. Offspring were screened for the presence of a null apo B allele and the human apo B transgene (apo B +/−, HuBTg) by PCR (5, 12).

Mature male mice (8 weeks old or older) of the indicated genotype were caged singly with normal female C57BL/6J mice for at least 4 months. Male mice failing to impregnate female mice within 4 months were considered infertile.

***In Vivo* Fertilization.** Immature female B6CBAF$_1$/J mice were superovulated with 2.5 units of pregnant mares' serum (Sigma) followed 48 hr later by 5 units of human chorionic gonadotropin (Steris, Mentor, OH). Immediately after the injection of human chorionic gonadotropin, female mice were mated with eight pairs of age-matched apo B +/− and control mice and five male apo B +/− mice containing the human apo B transgene. The next morning, female mice with vaginal plugs were sacrificed, and their ovaries and associated oviducts were removed to M16 medium (13) supplemented with 2 mg of hyaluronidase per ml (catalog no. H-3757; Sigma). The oocyte–cumulus complexes were released into this medium and incubated for 2–5 min at room temperature for dispersal of the cumulus cells. Oocytes were stored at 37°C for ≈3 hr. Zygotes were examined 3 hr later for fertilization, evidenced by the presence of two pronuclei.

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Abbreviations: apo B, apolipoprotein B; HuBTg, human apo B transgenic; RT, reverse transcription.

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Table 1. *In vivo* fertilization

Male genotype	*N*	Age, months	Eggs observed, no.	Eggs fertilized, no.	Fertilization, %
Apo B +/+	8	4.2 ± 0.9	175	129	74 ± 21*
Apo B +/−	8	4.4 ± 0.7	158	5	3 ± 4*†
Apo B +/−, HuBTg	5	3.5 ± 0.5	94	46	49 ± 32†

*Difference between apo B +/+ and +/+ mice. $P < 0.0001$, *t* test.
†Difference between apo B +/− and +/−, HuBTg mice. $P < 0.05$, *t* test.

***In Vitro* Fertilization.** Immature female B6D2F$_1$/J mice were superovulated as described above. Oocytes were collected from the oviduct and cumulus cells were similarly removed. Oocytes with identifiable first polar bodies were selected, washed three times in M16 medium, and loaded into organ culture dishes for insemination. For zona-free insemination, eggs were treated with acidified Tyrode's solution as described (14). For removal of sperm from apo B +/− and age-matched control males, the caudae epididymides with the entire associated vas deferentia were removed to culture dishes containing 2 ml of M16 medium. After all sperm was expressed from the vas deferentia, pressure was applied to the caudae with forceps, and sperm were released directly into the medium. The position of the forceps was periodically adjusted so as to remove the maximum number of sperm possible in each case. After removal, sperm were allowed to capacitate for 30 min. Before insemination, sperm were counted and the percentage of motile cells was determined. Inseminations were then performed at a final motile sperm concentration of 1×10^6 cells/ml. Oocytes were checked for fertilization 6–10 hr after insemination. Fertilization was confirmed by the presence of at least two pronuclei (many zona-free oocytes were polyspermic) and a second polar body. When no pronuclei were seen after 10 hr, oocytes were incubated overnight and examined the next morning to confirm the failure of fertilization and/or cleavage.

Evaluation of Sperm. Sperm were removed from the cauda epididymides as described above and incubated over a period of time. After 30 min of incubation in M16 medium, sperm were counted and the percentage of motile cells was determined. Sperm were then placed in 1.5-ml centrifuge tubes and incubated at 37°C until the following day. Periodically centrifuge tubes were gently inverted so as to create a homogenous suspension and sampled for a determination of the percentage of motile sperm and the total motile sperm counts. All counts were performed using a hemocytometer.

RNA Analysis by Reverse Transcription (RT)-PCR. Various tissues from apo B +/+, apo B +/−, and HuBTg mice (8 weeks old or older) were collected for RNA isolation. Epididymides from five males from each group were pooled prior to extraction for RNA isolation. Total cellular RNA was isolated using the guanidinium thiocyanate method (15). RT-PCRs were carried out using an RNA PCR kit (catalog no. N808-0143) obtained from Perkin–Elmer. The reaction was carried out according to the manufacturer's instructions. About 1 μg of RNA from the liver and intestine and 5 μg of RNA from the testes, epididymides, and spleen were used in the RT reaction. In the RT reaction, an antisense primer (100 pmol) specific for mouse apo B, human apo B, or random hexamer was used in the presence or absence of reverse transcriptase. The RT reaction was carried out in a final volume of 20 μl at 42°C for 1 hr, 99°C for 5 min, and then soaked at 4°C for 5 min in a DNA Thermal Cycler (Perkin–Elmer). For the PCR, 80 μl of PCR reagent mix containing both sense-strand primer (100 pmol) and the same antisense-strand primer (50 pmol) were used. Mouse apo B primers used were the same as those used for genotype screening of mice as described (5). Human apo B primers were as follows: 5′-CGCTGAGCCACGCGGTCAA-3′ (sense strand) and 5′-ACTTGAGTTCTGGAGTTAACTGCTC-3′ (antisense strand). Mouse β-actin primers were obtained from Stratagene. When mouse apo B primers or β-actin primers were used, PCRs were carried out at 94°C for 1 min, 57°C for 1 min, and then 72°C for 2 min, for a total of 35 cycles in a DNA Thermal Cycler (Perkin–Elmer). The same conditions were used for human apo B primers, except the annealing temperature was increased to 60°C.

RESULTS

Fertility Assessment and Fertilization *In Vivo*. The fertility of apo B +/− mice was recorded during the course of backcrossing (to C57BL/6J mice) and test mating. No apparent fertility problem was observed in female apo B +/− mice. In male mice, mating between male apo B +/− and wild-type female mice was documented by the presence of vaginal plugs in female mice. We found that although apo B +/− mice mated normally, only 40% (21/52) of the animals from the second backcross generation produced any offspring within the 4-month test period. Moreover, of the animals that produced progeny, litters resulted from <50% of documented matings (31 ± 17%). In contrast, all wild-type mice (6/6—i.e., 100%) tested were fertile. These data suggest genetic influence on the infertility phenotype, as a small number of male heterozygotes were not sterile.

Fertilization *in vivo* was dramatically impaired in male apo B +/− mice. As shown in Table 1, 74% (129/175) of eggs examined were fertilized by the sperm from wild-type mice, whereas only 3% (5/158) of eggs examined were fertilized by the sperm from apo B +/− mice. These results showed that the infertility of apo B +/− mice is most likely due to the inability of their sperm to fertilize oocytes.

In a separate experiment, heterozygotes were crossed with HuBTg mice. Five male offspring containing the human apo B transgene and one copy of the mutant mouse apo B gene (apo

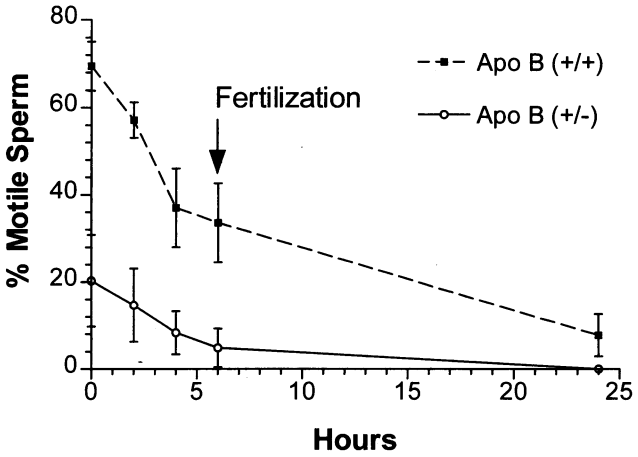

FIG. 1. Sperm survival time. Sperm were removed from the cauda epididymides and incubated over a period of time. The percent motile sperm at each time point is shown on the *y*-axis and the time of incubation is shown on the *x*-axis. The arrow indicates the 6-hr time point at which time fertilization often occurs after insemination. Error bars are based on the standard deviations. The filled square indicates apo B +/+ mice and the open circle indicates apo B +/− mice.

Table 2. *In vitro* fertilization

Male genotype	Zona pellucida	Experiments, no.	Eggs observed, no.	Eggs fertilized, no.	Fertilization, %
Apo B +/+	Intact	2	40	21	53
Apo B +/−	Intact	3	51	0	0
Apo B +/−	Free	3	37	31	84

B +/−, HuBTg) were subjected to similar fertility testing. As shown in Table 1, the percentage of fertilization increased to 49%. These results indicated that the defect in fertilization in apo B +/− mice could be at least partially corrected by the presence of the human apo B transgene.

Functional Abnormality of Sperm from Apo B +/− Mice. To characterize the sperm abnormality, sperm from six apo B +/− mice and age-matched controls were studied. The sperm counts of apo B +/− mice were mildly but significantly reduced ($4.5 \pm 1.0 \times 10^6$) compared with controls ($6.1 \pm 0.9 \times 10^6$; $P < 0.05$, t test). However, as shown in Fig. 1, the percentage of motile sperm was markedly reduced in the apo B +/− animals (20 ± 10.5%) compared with that of the wild-type controls (69.5 ± 5.6%). Sperm from apo B +/− mice also survived *in vitro* for a shorter period of time. Of the motile sperm present at the time of recovery from apo B +/− mice, 20% (i.e., 4.9% of the initial 20% motile sperm) remained motile after 6 hr of incubation, whereas 49% (i.e., 33.6% of the initial 69.5%) of the motile sperm retained motility in controls after this time (Fig. 1).

Oocyte fertilization was studied *in vitro*. As shown in Table 2, *in vitro* fertilization yielded no fertilized eggs (0/51) in three attempts with apo B +/− mice, while wild-type controls showed a fertilization rate of 53% (21/40). However, sperm from apo B +/− mice fertilized 84% (31/37) of eggs once the zona pellucida had been removed. We observed numerous sperm from apo B +/− mice binding to zona-intact eggs, binding comparable to that in the wild-type animals. However, these sperm lost their motility when observed 4–6 hr after binding. These results showed that sperm from apo B +/− mice were unable to penetrate the zona pellucida but that the interaction between sperm and egg was probably not directly affected. These results also showed that reduced sperm counts, percentage of motile sperm, and survival time of sperm all contribute to the infertility phenotype of apo B +/− mice.

Sperm binding to zona-free oocytes was also noted to be abnormal in apo B +/− mice. When zona-free mouse oocytes are inseminated, sperm binding is normally extensive until fertilization leads to establishment of a block to polyspermy. After fertilization, sperm binding progressively lessens, such that only a few sperm cells remain bound by the time pronuclei have formed (Fig. 2*A*). However, in the apo B +/− mice, sperm binding did not attenuate, even after pronuclei had clearly formed (Fig. 2*B*). This observation, recorded without exception in >30 fertilized eggs, suggests that apo B deficiency results in abnormal surface interaction between the sperm and egg.

Expression of Apo B in the Testis and Epididymis. The aforementioned abnormalities led us to determine if apo B mRNA was expressed in the testis and epididymis, where apo B expression had not been previously detected (16). RT-PCR was performed, and as shown in Fig. 3*A*, apo B mRNA was clearly detectable in both the testes (lane 4) and epididymides (lane 6) of wild-type mice. Uniform quality of all RNA samples was confirmed by appropriate controls. As shown in Fig. 3*A* (*Middle*), no bands were amplified, indicating that all RNA samples were free of DNA contamination and RNA was intact as mouse β-actin was amplified from every RNA sample (Fig. 3*A Bottom*). In contrast, apo B mRNA was barely detectable in the testes (lane 5) and epididymides (lane 7) of apo B +/− animals. Without exception, apo B mRNA was detected in the testis of apo B +/− mice at lower levels than that of controls. These data correlate reduced apo B mRNA levels in the testis and epididymis of apo B +/− mice with abnormal sperm function.

The identification of apo B transcripts in male reproductive tissues and the correction of infertility in these animals by the presence of the human apo B transgene would predict expression of the human transgene in the testis and epididymis of mice in which fertility was restored. To test this prediction, RT-PCR was carried out using human-specific apo B primers (Fig. 3*B*). Appropriate controls were carried out as above (Fig. 3*B*). These tests revealed the presence of human apo B mRNA in both the testis and epididymis of apo B transgenic mice. These data suggest that expression of human apo B in the testis and epididymis contributes to the correction of the infertility phenotype in apo B +/− mice containing the human apo B transgene.

DISCUSSION

Our identification of infertility in mice carrying a null allele for apo B and the correlation of this observation with abnormalities of sperm count, motility, and oocyte binding and fertili-

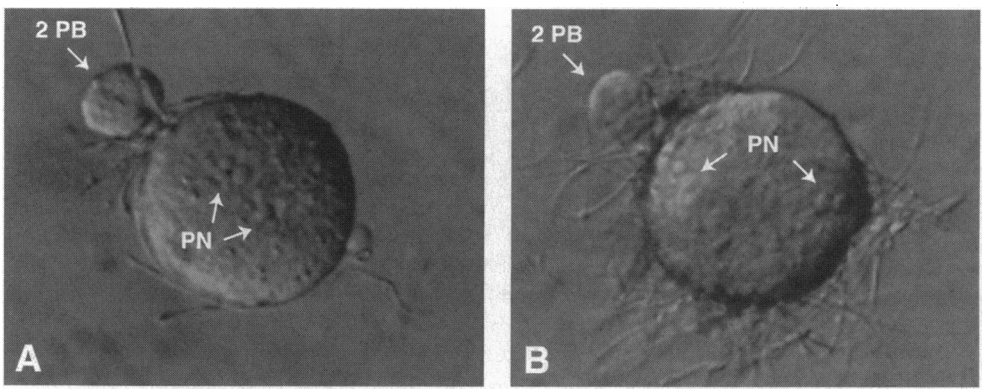

FIG. 2. Sperm binding to the zona-free fertilized egg. Sperm from either a wild-type (*A*) or an apo B +/− (*B*) mouse were incubated for 8 hr with zona-free eggs as described. Fertilized eggs were photographed under a light microscope. The second polar body is indicated as 2 PB and the pronuclei are indicated as PN.

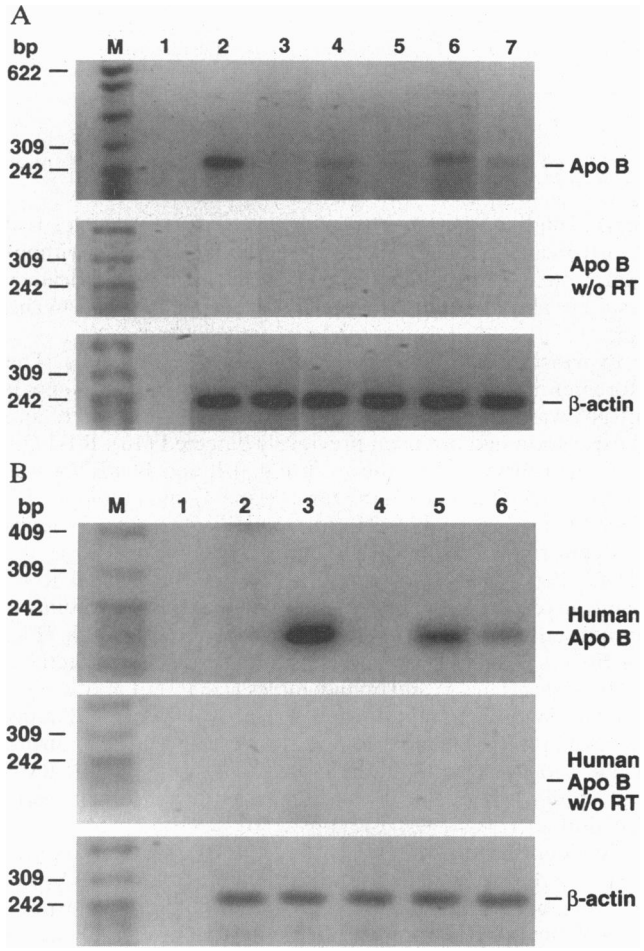

FIG. 3. Expression of the mouse apo B gene (*A*) and human apo B transgene (*B*) in the testis and epididymis. RNA samples from various tissues were subjected to RT-PCR analysis. (*A*, *Top*) Mouse apo B primers were used. (*Middle*) Mouse apo B primers were used but the reverse transcriptase was omitted in the RT reaction. (*Bottom*) Mouse β-actin primers were used. M, pBR322 *Msp*I-digested DNA fragments were used as the molecular mass markers. RNA samples used are shown from lanes 1 to 7. Lane 1, no RNA; lane 2, liver/wild-type; lane 3, spleen/wild-type; lane 4, testes/wild-type; lane 5, testes/apo B +/− mice; lane 6, cauda epididymides/wild-type; and lane 7, cauda epididymides/apo B +/− mice. (*B*, *Top*) Human apo B primers were used. (*Middle*) Human apo B primers were used but the reverse transcriptase was omitted in the RT reaction. (*Bottom*) Mouse β-actin primers were used. M, pBR322 *Msp*I-digested DNA fragments were used as the molecular mass markers. RNA samples used are shown from lanes 1 to 6. Lane 1, no RNA; lane 2, liver/wild-type; lane 3, liver/HuBTg; lane 4, intestine/HuBTg; lane 5, testes/HuBTg; and lane 6, cauda epididymides/HuBTg.

zation raise provocative questions concerning the role of apo B in the male reproductive process. That these findings have led to the identification of apo B mRNA in the testis and epididymis implicates this protein in spermatogenesis. This inference is strengthened still further by the detection of mRNA from a human apo B genomic clone in the testis of transgenic mice and the correction of infertility in heterozygous apo B knockout mice by the presence of an expressed human apo B transgene. The most reasonable interpretation of these results is that apo B performs an heretofore unrecognized function(s) in spermatogenesis. However, our data do not formally rule out the possibility that apo B deficiency outside the testis leads to physiological changes that secondarily impair male reproduction, nor that expression of this gene in the testis is directly related to gametogenesis. To address this question directly, experiments are currently in progress to identify and localize apo B in the testis and/or sex ducts.

Our characterization of sperm production and function in these animals has revealed a pattern of abnormalities that obscures which of the many steps in spermatogenesis is affected by the genetic alteration created in these animals. Sperm have reduced counts, motility, survivability, and penetration of the zona pellucida and bind abnormally to the oocyte surface. While it is difficult to confirm recovery of all sperm from the epididymides and vas deferentia for counting, the same recovery procedure was utilized for all animals, and results showed a reduction in count that was statistically significant. Such a diversity of abnormalities suggests that if only one step of spermatogenesis is affected by apo B, it resides relatively early in spermatogenesis. Problems with epididymal or postejaculatory maturation, although not to be ruled out, would not be expected to reduce sperm counts, nor would deficiencies in capacitation and/or the acrosome reaction. However, since sperm production and postmeiotic maturation is critically dependent upon interaction with the Sertoli cell (17), a defect in this interaction could lead to the multiple defects manifested in sperm samples from these mice. We have examined both the testis and spermatocytes from the infertile mice by transmission electron microscopy, and no obvious abnormalities were apparent (data not shown). Of course, the possibility that more than one step in spermatogenesis is compromised by apo B deficiency has not been ruled out. While binding to the zona pellucida was observed when zona intact oocytes were inseminated, it is clear that this binding does not lead to zona penetration. It is therefore possible that all fertilizable sperm in these animals undergo a premature acrosome reaction. In this circumstance, zona penetration would be blocked but oocyte penetration would not. A premature acrosome reaction cannot explain all the findings, however. The maintenance of aggressive binding to zona-free eggs even after fertilization (Fig. 2) certainly indicates that other sperm surface abnormalities exist.

Male infertility in humans undoubtedly has numerous causes. However, in very large numbers of individuals, infertility is idiopathic. Chromosomal abnormalities, such as Klinefelter syndrome, account for some of these problems, as do environmental factors, such as infection (18) or alcohol abuse (19). While mutations of individual genes almost certainly play a role as well, such genes have not been identified in large numbers. The findings here indicate that the apo B locus deserves investigation for a possible causative role in male infertility. Moreover, as the combination of abnormalities displayed by infertile heterozygous apo B knockout mice is not dissimilar from that seen in many infertile human males (20, 21), this locus could play a significant role in this common medical problem.

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