Fertility Defects in Mice Expressing the L68Q Variant of Human Cystatin C

A ROLE FOR AMYLOID IN MALE INFERTILITY*

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Abstract:

Hereditary cystatin C amyloid angiopathy is an autosomal dominant disorder in which a variant form of cystatin C (L68Q) readily forms amyloid deposits in cerebral arteries in affected individuals resulting in early death. L68Q protein deposits in human cystatin C amyloid angiopathy patients have also been found in tissues outside of the brain including the testis, suggesting possible effects on fertility. Heterozygous transgenic mice (L68Q) that express the human L68Q variant of cystatin C under the control of the mouse cystatin C promoter were unable to generate offspring, suggesting the presence of L68Q cystatin C amyloid affected sperm function. In vitro studies showed that epididymal spermatozoa from L68Q mice were unable to fertilize oocytes and exhibited poor sperm motility. Furthermore, spermatozoa from L68Q mice exhibited reduced cell viability compared with wild type (WT) spermatozoa and often were detected in large agglutinated clumps. Examination of the epididymal fluid and spermatozoa from L68Q mice showed increased levels and distinct forms of cystatin C amyloid that were not present in WT mice. The addition of epididymal fluid from L68Q mice to WT spermatozoa resulted in a recapitulation of the L68Q phenotype in that WT spermatozoa showed reduced cell viability and motility compared with WT spermatozoa incubated in epididymal fluid from WT mice. L68Q epididymal fluid that was depleted of cystatin C amyloids, however, did not impair the motility of WT spermatozoa. Taken together these studies suggest that amyloids in the epididymal fluid can be cytotoxic to the maturing spermatozoa resulting in male infertility.

Background: The L68Q variant of cystatin C is highly amyloidogenic forming aggregates in individuals with HCCAA.

Results: Spermatozoa from mice expressing human L68Q cystatin C exhibit fertility defects and increased levels of amyloid.

Conclusion: L68Q epididymal fluid containing cystatin C amyloid is harmful for sperm function.

Significance: Amyloid in the reproductive tract may contribute to male factor infertility.

Human cystatin C has been implicated in neurodegenerative diseases and in particular Alzheimer disease, as suggested by the genetic linkage of a cystatin C polymorphism with late-onset AD (12, 13) and that cystatin C colocalizes with amyloid-β plaques associated with Alzheimer disease (14). However, cystatin C seems to play a protective role as in vitro studies showed that cystatin C association can inhibit amyloid-β fibril formation (15–17) and in vivo cystatin C inhibited the deposition of amyloid-β in several amyloid precursor protein mouse models (18–20). Interestingly, cystatin C itself has also been shown to self-aggregate and form amyloid fibrils (21). Cystatin C crystallized as a domain-swapped dimer in which the tertiary structure elements of the monomeric-fold were exchanged between two participating monomers (22, 23). Domain swapping has been observed in several amyloidogenic proteins including prion protein and β-2-microglobulin and, therefore, has been proposed as a mechanism for the formation of amyloid fibrils (24–27). Indeed, prevention of domain swapping by the generation of stabilized disulfide bonds or a hinge loop mutation inhibited cystatin C dimerization and formation of amyloid fibrils (28–30).

Cystatin C (Cst3) belongs to the family 2 of the cystatin superfamily of reversible inhibitors of cysteine proteases of the papain and legumain families (1). Cystatin C is a 13-kDa secreted protein that is broadly expressed and is present in all human biological fluids and tissues examined with very high concentrations in seminal plasma and cerebrospinal fluid (2). As a potent inhibitor of C1 family of cysteine proteases including the lysosomal cysteine proteases cathepsins B, H, L, and S, cystatin C is thought to play a protective role against tissue destruction, whereas other roles in bone resorption, tumor metastasis, protein processing, and parasitic infections have also been proposed (3–7). Cst3 resides on human chromosome 20 and mouse chromosome 2, where it clusters with several other cystatin family 2 members including members of a reproductive subgroup comprising Cst8 (cystatin-related epididymal spermaticogenic (CRES)3), Cst9 (testatin), Cst11 (CRES2), Cst12 (CRES3), cystatin E2, and others (8–11).

The abbreviations used are: CRES, cystatin-related epididymal spermaticogenic; AD, Alzheimer disease; HCCAA, human cystatin C amyloid angiopathy; K’som, K+-modified simplex optimized medium; MTT, thiazolyl blue tetrazolium bromide; CASA, computer-assisted sperm analysis; RT, room temperature; PAD, protein aggregation disease.

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In addition to amyloid formation in wild type cystatin C, a single point mutation (leucine 68 to glutamine, L68Q) in human cystatin C results in a highly unstable and highly amyloidogenic protein that readily forms an amyloid at 37 °C (31–33). Patients with this hereditary form of cystatin C amyloid angiopathy (HCCAA), also known as hereditary cerebral amyloid with amyloidosis, Icelandic type, die in their 30–40s as a result of cerebral hemorrhage due to L68Q cystatin C deposits in the cerebral arteries (34, 35). In HCCAA patients cystatin C deposits have also been found outside the central nervous system including the testis (36, 37), and anecdotal evidence suggests that affected males have fertility problems. The HCCAA disease is autosomal dominant with patients expressing both the wild type and the L68Q variant form of human cystatin C (38).

To develop a mouse model for HCCAA, transgenic mice expressing the human L68Q cystatin C in addition to mouse cystatin C proteins were generated. Heterozygous L68Q male mice were unable to generate offspring, suggesting that L68Q cystatin C amyloid may be detrimental to reproductive function. The present studies were carried out to identify the fertility defect in L68Q male mice and to examine whether human L68Q cystatin C amyloid present in epididymal fluid may be detrimental for sperm function and thus play a causative role in male infertility.

**EXPERIMENTAL PROCEDURES**

**Animals**—CD1 strain male (retired breeders) and female mice were purchased from Charles River Laboratories. The C57 129Sv/B6 heterozygous transgenic L68Q and WT mice were bred in-house. Mice were maintained under a constant 12-h light:12-h dark photoperiod with food and water ad libitum. All animal studies were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

**Generation of Transgenic Mice**—Because in previous studies constructs based on a cDNA segment for human L68Q cystatin C to replace mouse cystatin C were found to be nonfunctional in mice (5), in the present study constructs were assembled from human genomic cystatin C segments including exon and intron sequences. Briefly, the original targeting plasmid, pTG-6 (5), was digested with NotI, and an 11-kb fragment was subcloned into pBluescript SK. A 700-bp SfiI/EcoRI fragment containing the human cystatin C cDNA was excised and replaced with a 2.2-kb fragment of human cystatin C gene containing the promoter, exon 1, and most of intron 1, resulting in a 15.2-kb plasmid called pKH12. The remaining part of the human cystatin C gene including the mutated codon 68 in exon 2 was generated by PCR amplification using genomic DNA from a blood sample from a HCCAA patient as template and subcloned into pKH12, resulting in a plasmid with all the introns and exons of the human cystatin C gene but with the codon for Leu68 replaced by that for Gln (causing the L68Q mutation). The NotI fragments of 14.8 kb were then excised from pKH12 and ligated with the remaining 6-kb NotI fragment of pTG-6 downstream of the mouse cystatin C gene promoter with the resulting plasmid called pKH16. The targeting vector was excised and electroporated into E14.1 129/Sv ES cells using a Bio-Rad Gene Pulser. After selection in G418, the surviving clones were expanded for PCR analysis as well as examined by fluorescence in situ hybridization to confirm the localization of the L68Q construct to the cystatin locus on mouse chromosome 2 as described previously (8). The correctly targeted 129/Sv ES clones were microinjected into C57Bl/6J (B6) mouse blastocysts and implanted into pseudopregnant mice. The resulting chimeric mice were mated to B6 mice to generate germ line transmission. Genomic DNA from mouse tail snips was isolated, and mice were genotyped by PCR using the XNAT kit (Sigma) and primers 5'-TGTAAGAAACTGACAGAACATGTGCATC to detect human cystatin C. PCR conditions were 95 °C for 3 min, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min for 35 cycles followed by 72 °C for 7 min.

**In Vivo Breeding**—WT and L68Q heterozygous transgenic male mice (12–20 weeks of age) were paired with WT female mice (12 weeks of age) in a 1:1 ratio. Females were checked each day and placed in separate cages when pregnancy was detected. Litter size was determined by counting both live and dead pups on the day of delivery or the following day.

**In Vitro Fertilization Studies**—All reagents used were from Sigma. Unless otherwise indicated, the medium used for all fertilization experiments was KSOM (K+-modified simplex optimized medium) (39) containing 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH2PO4, 0.20 mM MgSO4·7H2O, 10 mM sodium lactate, 0.20 mM glucose, 0.20 mM sodium pyruvate, 25 mM NaHCO3, 1.71 mM CaCl2, 1.0 mM glutamine, 0.01 mM EDTA, 50 µg/ml gentamycin, and 0.3% bovine serum albumin (BSA; embryo-tested, Sigma). KSOM with Hapes (KSOM-Hapes) also contained 20 mM Hapes, and NaHCO3 was reduced to 4 mM. For an experiment, incomplete KSOM was preequilibrated overnight at 37 °C in a humidified water-jacketed incubator under 5% CO2. The next day CaCl2 and BSA were added, the pH adjusted to 7.4, and the medium was sterile-filtered and placed in the CO2 incubator with the cap loose to allow gas exchange.

**In vitro fertilization** sperm–zona pellucida binding, and sperm–zona free fertilization were carried out as previously described except that KSOM buffer was used in place of KREBS (40). Briefly, cauda epididymides isolated from WT and L68Q mice were placed into KSOM and punctured with a 26-gauge needle to allow spermatozoa to disperse. Spermatozoa were capacitated (20 × 106 spermatozoa/ml) in KSOM for 1.5 h at 37 °C in a humidified water-jacketed incubator under 5% CO2. The next day CaCl2 and BSA were added, the pH adjusted to 7.4, and the medium was sterile-filtered and placed in the CO2 incubator with the cap loose to allow gas exchange.

**Sperm Motility Analysis**—Motility of WT and L68Q spermatozoa capacitated in KSOM for 30, 60, 90, and 180 min was assessed using a computer-assisted sperm analysis (CASA; HTM-CEROS Version 12; Hamilton Thorne Research, Beverly, MA). Sperm concentrations were adjusted to 5–10 × 106 spermatozoa/ml, and 12 µl of sample were loaded on a prewarmed counting chamber and the samples immediately analyzed. Ten
fields were examined on duplicate slides for each time point to determine the percentage motile and percentage progressively motile spermatozoa.

Sperm Assays—Cauda epididymides from age-matched WT and L68Q mice were placed in 37 °C Dulbecco’s PBS or Hanks’ balanced salt solution in a 35-mm Falcon dish, punctured with a needle, and placed in the CO₂ incubator with 5% CO₂ for 15 min at 37 °C to allow sperm to disperse. Epididymides were removed, and the sperm suspension was centrifuged at 500 × g for 5 min to pellet the spermatozoa. The sperm pellet was gently washed free of luminal fluid proteins by resuspension in warm PBS and centrifugation at 500 × g for 5 min. The sperm pellet was then resuspended in a small volume of PBS and kept at 37 °C, whereas an aliquot was counted with a hemacytometer.

For sperm viability assays, 5 × 10⁶ WT and L68Q sperm were immediately diluted into 37 °C PBS, and 1/10 volume of SDS-PAGE loading buffer (thiazolyl blue tetrazolium bromide) was added following the manufacturer’s instructions (Sigma), and samples were incubated at 37 °C for 30 min. Control samples contained PBS and MTT only. The samples were then removed, 1/10 volume of MTT solubilization buffer containing 10% Triton X-100 in acidic isopropyl alcohol was added, and the samples were incubated in a 37 °C oven for 1 h to allow solubilization of MTT crystals. The samples were centrifuged at 5 min at 15000 × g in a microcentrifuge, and the supernatant was examined at A₅₇₀ and A₆₅₀ using a Tecan Infinite M1000 plate reader (Tecan, San Jose, CA). The A₅₇₀-A₆₅₀ was determined, and the sample with the highest relative absorbance was normalized to 1 to control for variability in spermatozoa between experiments.

For sperm extractions, 1 × 10⁶ of washed cauda epididymal spermatozoa from WT and L68Q mice were resuspended in 30 μl of low salt buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA) and extracted on ice for 1 h. Samples were then centrifuged at 15,000 × g for 5 min, and a proportion of the resulting supernatant was resuspended in Laemmli buffer with β-mercaptoethanol for Western blot analysis, whereas the majority of the supernatant was used for protein aggregation disease (PAD) pulldown experiments. The low salt-extracted spermatozoa were then incubated in 1% Triton X-100 in PBS for 1 h on ice and centrifuged at 15,000 × g. A proportion of the supernatant was removed for Western blot analysis, and the balance was incubated with PAD ligand in pulldown experiments. Samples were heated at 95 °C for 5 min before loading on SDS-PAGE gels and Western blot analysis as described below.

To examine sperm agglutination, paraformaldehyde (4% in PBS) (EMS Sciences, Hatfield, PA) was added to 1 × 10⁶ sperm from WT and L68Q mice in PBS and incubated at 4 °C for 30 min. Aliquots were then placed on a microscope slide, cover-slipped, and examined with a Zeiss Axiosvert 200 inverted microscope.

Isolation of Epididymal Luminal Fluid—Epididymides from WT and L68Q mice were dissected free of fat and sectioned into 5 regions including proximal caput (1), midcaput (2), distal caput (3), corpus (4), and cauda (5) as described previously (41). In some experiments, regions 1–3 were combined and regions 4–5 were combined to yield caput and corpus-cauda extracts. Tissues were then placed in PBS on ice and minced to release spermatozoa and luminal fluid proteins. After dispersion for 15 min, the sperm suspensions were then centrifuged at 500 × g for 5 min to pellet spermatozoa. The resulting supernatant containing luminal fluid proteins was then either used directly in experiments or sequentially centrifuged at 5000 × g, 10 min (pellet 2), 15,000 × g, 10 min (pellet 3), and at 250,000 × g for 1 h (pellet 4) to pellet insoluble material of varying molecular mass. The pellets were then resuspended in PBS.

Sperm/Luminal Fluid Coincubation—Cauda epididymides were removed from WT and L68Q mice and punctured in warm PBS to disperse spermatozoa and luminal proteins as described above. The sperm suspension was centrifuged at 500 × g for 5 min to pellet the spermatozoa. The resulting supernatant containing luminal fluid proteins was then centrifuged again at 500 × g to pellet any remaining spermatozoa, and the luminal fluid was kept in a 37 °C heater block; a small aliquot was removed for quantitation by spectrophotometric measurement at A₂₈₀ (Nanodrop ND-1000, ThermoScientific, Waltham, MA). To deplete amyloids from the luminal fluid, it was centrifuged at 250,000 × g for 1 h, and the protein concentration of the resulting supernatant was determined by A₂₈₀. Luminal fluid and supernatant protein concentrations were adjusted to the lowest concentration by diluting with PBS. Equal amounts of luminal fluid and supernatant protein (~1–1.6 mg/ml) from either the WT or L68Q cauda epididymis were then incubated with 5 × 10⁵ WT cauda epididymal spermatozoa at 37 °C. To examine the effect of luminal fluid and supernatant on sperm motility, samples were examined for percent progressive motility by visual examination with an AxioVision microscope. Approximately 100+ sperm/sample were examined, and any forward movement was considered to be progressive. Spermatozoa that only showed twitching were not considered to have progressive motility. Visual assessment of motility was required as the CASA system was not available to us during this study. To examine the effect of luminal fluid on sperm viability, 1/10 volume of MTT was added at the start of the sperm/luminal fluid coincubation, and samples were removed after various times to examine MTT crystal formation as described above.

ELISA—Human cystatin C immunoreactivity in epididymal fluid from WT and L68Q mice was quantified using an enzyme-linked immunosorbent assay as described previously (42). Briefly, a microtiter plate was coated with a polyclonal rabbit anti-human cystatin C antibody overnight, washed, and then incubated with 1:200 dilution of epididymal fluid isolated from each of the five regions of the mouse epididymis at room temperature for 1 h. Brain and testis lysates from WT and L68Q mice were also examined. The plates were then washed and incubated for 1 h with a mouse monoclonal anti-human cystatin C antibody that specifically recognizes human but not mouse cystatin C (5). The plate was washed again, and HRP-labeled polyclonal rabbit antibodies to mouse immunoglobulins were added. After washing, the substrate 2,2'-azido-bis-(3-ethylbenzthiazoline)sulfonic acid (ABTS) was added and the absorbance measured after 45 min at 405 nm.

Electrophoresis/Western Blotting—Proteins were separated by 1% agarose gel electrophoresis in 75 mM barbituric acid, 2 mM EDTA, pH 8.6 (43), or by SDS-PAGE using 15% Tris-glycine Criterion gels (Bio-Rad) (40). After electrophoresis the proteins were transferred to PVDF membrane (Immobilon-P, Millipore, Billerica, MA) by press blotting for agarose gels or
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Using a Bio-Rad transfer cell 1.5 h at 100 V for SDS-PAGE gels. The membranes were incubated in 3% milk in TBST (0.2% Tween 20) for 1 h at room temperature followed by incubation with a rabbit anti-human cystatin C IgG (Dako, Carpinteria, CA) (1:8000) in milk buffer overnight at 4 °C. This antibody recognizes both mouse and human L68Q cystatin C. The membranes were washed in TBST followed by incubation with a goat anti-rabbit HRP secondary antibody (SDS-PAGE gels, 1:40,000; Invitrogen) or a swine anti-rabbit HRP secondary antibody (agarose gels, 1:30,000) in 3% milk buffer for 2 h at room temperature. The membranes were washed extensively in TBST and then incubated with SuperSignal West Pico chemiluminescent substrate (ThermoScientific) for 5 min followed by exposure to film.

Immunofluorescence Analysis and Amyloid Staining—Epididymal pellet 4 (4 μl) was spread on Superfrost Plus slides (ThermoScientific) within a small square drawn with a Pap pen and allowed to air-dry. Slides were then placed in a humid chamber and fixed in 3.7% formaldehyde in PBS for 30 min at RT followed by incubation in 1% Triton X-100 for 3 min. The slides were washed in PBST (PBS containing 0.1% Tween 20) 3×, and 100 μl of rabbit anti-human cystatin C antibody (Dako) or rabbit IgG (Pierce) diluted 1:1000 in 10% goat serum, PBST was added to each slide and incubated overnight at 4 °C. The next day slides were washed 5× in PBST and incubated with a goat anti-rabbit AlexaFluor-594 secondary antibody (Invitrogen) diluted 1:250 in 10% goat serum, PBST for 2 h at RT in the dark. Slides were then washed 5× in PBST, 3× in PBS, and 2× in distilled H2O, and then put into 0.1% Thioflavin S (Sigma) in water for 2 h staining in the dark. Slides were washed 3× distilled H2O, 2× in 50% EtOH, and 2× in distilled H2O before mounting with Vectamount AQ (Vector Laboratories, Burlingame, CA). To break apart higher ordered structures, some slides were first incubated in 90% DMSO in PBS for 90 min at RT and then washed 5× in PBS before fixation. In other experiments, epididymal pellet 4 was spread on slides and air-dried as described above. Slides were then washed in TBST 4× 2 min each and then blocked in 100% goat serum 1 h at RT followed by incubation with a rabbit anti-amyloid fibril OC antibody (Millipore) or normal rabbit serum in 1% BSA, TBS overnight at 4 °C. Slides were then washed 5× for 2 min each in TBST followed by blocking in 100% goat serum for 1 h at RT and then incubation with a goat anti-rabbit AlexaFluor-594 secondary antibody diluted 1:250 in 1% BSA, TBS for 30 min at RT in the dark. Slides were washed in TBST 3× for 2 min each, low salt TBS 2× for 2 min, and then rinsed in distilled H2O and mounted with Fluormount G (Southern Biotech, Birmingham, AL). Slides were examined for AlexaFluor-594 fluorescence and for Thioflavin S with excitation at 440 and emission at 485 using a Zeiss Axiovert 200 microscope with an attached digital camera.

Negative Stain Electron Microscopy—Pellet 4 (5 μl) was spotted on to Formvar/carbon-coated 200 mesh nickel grids (Ted Pella, Redding, CA) for 1 min. The sample was wicked off with filter paper, washed for 1 min with water, stained with 2% uranyl acetate for 1 min, and washed again with water for 1 min. Samples were examined with a Hitachi 81000 electron microscope operating at an excitation voltage of 75 kV.

RESULTS

L68Q Transgenic Mouse Model—Patients with the autosomal dominant disease HCCA are heterozygous in that one allele expresses normal cystatin C, whereas the other expresses the L68Q mutant form of cystatin C. To develop a mouse model for this disease, transgenic mice expressing the L68Q form of human cystatin C under the control of the mouse cystatin C promoter were generated. Fluorescent in situ hybridization confirmed the human L68Q cystatin C construct localized to the cystatin locus on mouse chromosome 2 as expected (data not shown). As shown below (see Fig. 4), both mouse and human cystatin C proteins were expressed in the heterozygous L68Q mouse, whereas only mouse cystatin C was present in the WT mice. Together, these experiments suggested the transgenic mouse was an appropriate model for HCCA.

Mating Studies—To examine the fertility of the transgenic mice, male L68Q and WT mice were mated with female WT mice, and successful fertilization events were determined by the delivery of pups. As shown in Fig. 1, WT male mice readily generated offspring with an average of 4.6 pups/litter in 28 matings examining 17 different males, whereas L68Q males generated 0.2 pups/litter in 24 matings examining 15 different males (p < 0.001). Of the 28 WT male × WT female matings, only 5 of the WT females did not deliver pups, which may reflect a fertility problem with the female and which reduced the average number of pups/litter to 4.6. When these females were excluded from the analysis, the average number of pups/litter for WT male × WT female matings was 7.6. Of the 24 L68Q male × WT female matings, 20 of the females did not produce pups, and of the 4 females that did produce pups there were only 1–2 pups/litter. In all mating experiments females were left with males until visible signs of pregnancy were evident and for females mated with WT males the average time to an obvious pregnancy was 15 days. Because most of the females paired with
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**FIGURE 1. Fertility of WT and L68Q male mice.** Male mice were housed with WT female mice, and the number of live and dead offspring was determined on the day or the day after delivery. WT males: n = 28 matings examining 17 different males; L68Q males: n = 24 matings examining 24 different males. Values represent the mean ± S.E. * p < 0.001.

L68Q males showed no signs of pregnancy after 14 days, they were left with the males for extended periods of time including up to 40–55 days, and still no pregnancies occurred. Although female mice were not checked for copulatory plugs, WT and L68Q males exhibited normal mounting behavior upon introduction to the female mice. Together these studies demonstrated that the L68Q male mice were subfertile, and therefore, in vitro studies were carried out next to identify the fertility defect.

**In Vitro Fertility and Motility Studies**—To examine the fertility of WT and L68Q mice in vitro, capacitated cauda epididymal spermatozoa from WT and L68Q males were added to cumulus oocyte complexes from CD-1 female mice, and the percentage of oocytes that were fertilized was determined was determined. As an additional control, cumulus oocyte complexes were also inseminated with capacitated cauda epididymal spermatozoa from CD-1 males. As shown in Fig. 2A, spermatozoa from L68Q mice were unable to fertilize cumulus oocyte complexes, in contrast to spermatozoa from CD-1 and WT males, which fertilized 60–80% of the oocytes (p < 0.01). Similarly, spermatozoa from L68Q mice were unable to fertilize oocytes after removal of the cumulus cells (Fig. 2B) and did not bind as efficiently to the zona pellucida as spermatozoa from WT and CD-1 mice (p < 0.01) (Fig. 2C). However, when the zona pellucida was removed from the oocytes, spermatozoa from L68Q mice were able to fertilize oocytes and generate blastocysts as efficiently as spermatozoa from WT and CD-1 mice (Fig. 2, D and E). A CASA showed that spermatozoa from the L68Q mice exhibited extremely poor motility with little to no forward progression compared with spermatozoa from WT mice (p < 0.05) (Fig. 2F). Together these data suggested that because of their poor motility L68Q spermatozoa were unable to fertilize oocytes unless some of the barriers to fertilization were removed.

**Sperm Viability and Agglutination**—Because spermatozoa from L68Q mice showed reduced motility, studies were carried out next to determine if the poor motility could reflect dying spermatozoa. Using the MTT assay to measure mitochondrial function, L68Q cauda epididymal spermatozoa were found to be significantly less viable after 30 min of incubation in buffer than WT spermatozoa (p < 0.05) (Fig. 3A). Because spermatozoa are heterogeneous, the decrease in viability may represent a small, yet significant, uniform effect across the entire L68Q sperm population or could be due to large decreases in sperm viability in only a discrete population of spermatozoa. Nevertheless, these studies showed that compared with WT spermatozoa, the L68Q spermatozoa were less healthy.

L68Q cauda epididymal spermatozoa were also often present in large agglutinated clumps after dispersion into buffer and were not detected in WT sperm preparations (Fig. 3B). Furthermore, in the smaller agglutinated clumps L68Q spermatozoa were sometimes observed sticking to amorphous material that was not evident in suspensions of WT spermatozoa.

**Cystatin C Protein in Mouse Epididymal Fluid**—To examine cystatin C protein, luminal fluid was isolated from the five regions of the WT and L68Q mouse epididymis including proximal caput (1), midcaput (2), distal caput (3), corpus (4), and cauda (5), and Western blot analysis was performed using a rabbit anti-human cystatin C antibody that detects both human and mouse cystatin C (Fig. 4A). In the WT mice an ~13-kDa cystatin C protein was detected in the luminal fluid with higher levels in the cauda as compared with the caput (regions 1–3), which may reflect the accumulation of cystatin C protein in the fluid as it moves from the proximal to the distal regions of the epididymis. Luminal fluid isolated from the epididymides from L68Q mice contained higher levels of cystatin C in both the caput and cauda regions compared with WT mice likely representative of the presence of human cystatin C protein in addition to the mouse cystatin C (Fig. 4A).

Because mouse and human cystatin C are of similar molecular mass on SDS-PAGE, next we used an ELISA with a mouse monoclonal antibody that specifically detected human cystatin C (both wild type and L68Q variant) (5) but not mouse cystatin C. These studies showed that the epididymal luminal fluid as well as brain and testis lysates from the L68Q but not the WT mice contained human cystatin C (Fig. 4B). Agarose gel electrophoresis followed by Western blot analysis to detect both mouse and human cystatin C was also carried out that allowed separation of the various forms of cystatin C based on charge. As shown in Fig. 4C, several forms of mouse cystatin C were detected in the luminal fluid from the WT mouse epididymis, which is consistent with the known presence of differentially glycosylated forms of cystatin C in rodents (44). In luminal fluid from the L68Q mouse epididymis, much higher levels of cystatin C were present with the human cystatin C migrating to the same position as a form of mouse cystatin C. A comparison of the form of mouse cystatin C (most positively charged) that did not overlap with that of the human L68Q cystatin C showed similar levels in the epididymal fluid from the WT and L68Q mice, indicating that expression of human L68Q cystatin C did not result in an up-regulation of mouse cystatin C (Fig. 4C). Together these studies demonstrated that the higher levels of total cystatin C as detected by SDS-PAGE in L68Q samples is due to the presence of the human L68Q cystatin C protein. We also observed high levels of what has been previously described as high molecular weight human cystatin C complexes that were only present in the luminal fluid and tissues from the L68Q mice (45). These high molecular weight structures may represent aggregates of the highly amyloidogenic human L68Q cystatin C. These structures were not detected by SDS-PAGE, suggesting that they may be SDS-sensitive or alternatively too large to enter into standard SDS-PAGE gels.
Increased Cystatin C Amyloid in the L68Q Mouse Epididymal Lumen—We previously demonstrated that CRES (Cst8) protein, a reproductive-specific member of the family 2 cystatins, forms a nonpathological amyloid within the mouse epididymal lumen, suggesting a functional role for cystatin amyloid structures in sperm maturation and epididymal function (41). Furthermore, we have shown that a population of CRES amyloid is present in a film-like insoluble material that pellets at 250,000 g (pellet 4) (41). Because human L68Q cystatin C is highly amyloidogenic and is associated with disease, we hypothesized that the fertility defects in the L68Q mice could be due to increased levels of amyloid or altered forms of amyloid in the epididymal lumen that resulted in cytotoxic effects on sperm function.

To begin to address this, we first examined whether cystatin C amyloid was present within the epididymal lumen and if there were increased amounts of cystatin C amyloid in the epididymal lumen from the L68Q mice compared with that in WT mice. Using the polyclonal cystatin C antibody that recognizes both mouse and human cystatin C and immunofluorescence analysis, we determined that cystatin C colocalized with the Thioflavin S-positive film-like material (pellet 4) in the caput and cauda epididymal lumen from WT mice, suggesting that a proportion of mouse cystatin C contributes to the formation of the functional epididymal amyloid structure (Fig. 5A). More cystatin C immunoreactivity, however, was present in the film-like amyloid isolated from the L68Q mouse caput epididymal fluid compared with that in the WT epididymal fluid (Fig. 5A). Furthermore, in the L68Q epididymis, cystatin C was also present in punctate thioflavin S-positive structures of various size that were associated with the film-like material but were not as pronounced in the WT epididymis, suggesting additional and different populations of amyloid were present (Fig. 5A). In the cauda epididymis from the L68Q mice, although increased levels of cystatin C were detected, the film-like material was extremely dense with decreased Thioflavin S staining compared with that in WT mice, implying either reduced levels of amyloid or that due to the presence of additional amyloid higher ordered structures formed such that it prevented Thioflavin S from binding (Fig. 5A). Despite several attempts, we were unsuccessful in using the mouse monoclonal antibody to examine only human cystatin C by immunofluorescence or Western blot analysis (data not shown).

Immunofluorescence studies using the OC conformation-dependent antibody that recognizes fibrillar forms of amyloid showed specific staining in the film-like material isolated from the caput and cauda epididymis, confirming the presence of amyloid in this structure (Fig. 5B). As expected, the OC antibody detected amyloid in both the WT and L68Q epididymis. However, more punctate forms of OC positive amyloid were present in the L68Q film-like material.

To confirm there were increased levels of cystatin C amyloid in the epididymis from the L68Q mouse, the insoluble material pelleted after 250,000 × g (pellet 4) was incubated with the PAD ligand, which binds amyloid structures but not natively folded or monomeric proteins. The eluted proteins were then examined for cystatin C by SDS-PAGE and Western blot analysis. As shown in Fig. 5C and as shown previously in Fig. 4A, more
Cystatin C was present in the starting luminal fluid preparation from both the caput and cauda epididymis from the L68Q mice compared with that in the control WT mice reflecting the additional presence of human L68Q cystatin C. After incubation with the PAD ligand, only very low levels of cystatin C amyloid were bound from either sample, although more cystatin C amyloid was detected in the L68Q samples. Because DMSO has been shown to break hydrogen bonds and reverse higher ordered amyloid structures (46), the pellet fraction was incubated with 90% DMSO for 20 min before PAD ligand binding. Exposure of the pellet fraction to DMSO resulted in a large increase in the amount of cystatin C amyloid that was detected in both WT and L68Q samples with 5–10-fold more cystatin C amyloid present in the L68Q than in the WT pellet fraction. We have previously determined that DMSO does not alter the binding specificity of the PAD ligand such that monomeric or natively folded proteins can bind. The increase in the amount of cystatin C amyloid detected after DMSO exposure suggested that the epididymal amyloids are in higher ordered structures, especially in the L68Q epididymal lumen, and are partially reversed allowing additional amyloids to bind to the PAD ligand. This is consistent with the presence of the extremely dense film-like material in the cauda epididymal fluid from the L68Q mice (Fig. 5A).

To determine if the film-like amyloid containing cystatin C was altered with DMSO treatment, immunofluorescence analysis was carried out on pellet 4 after exposure to DMSO for varying lengths of time. As shown in Fig. 5D, the film-like material was solubilized within 20 min after DMSO treatment resulting in a loss of cystatin C immunofluorescence and thioflavin S staining. These studies suggest that amyloids present in this film are reversed upon DMSO treatment yet still retain sufficient β-sheet structure, possibly as soluble oligomers or protofibrils, to allow binding to the PAD ligand. In contrast to the film, the cystatin C/thioflavin S-positive punctate structures that were in both the WT and L68Q epididymal fluid appeared more resistant to DMSO treatment. However, after 90 min of DMSO, only the cystatin C punctate structures in the L68Q epididymal fluid remained, suggesting a more resistant amyloid structure than those in the WT epididymal fluid (Fig. 5D).

Negative stain electron microscopy was carried out to examine the structures in pellet 4 isolated from caput and cauda
epididymal fluid from WT and L68Q mice. As shown in Fig. 6, fibrillar structures characteristic of amyloid were present in both caput and cauda samples from WT and L68Q mice. However, pellet 4 from L68Q fluid contained more fibrils as well as large, dense structures that appeared to represent bundles of fibrils compared with those present in WT pellet 4 (Fig. 6). Together these studies show that there is more cystatin C amyloid as well as different amyloid structures present in the epididymal fluid from the L68Q mice compared with that in WT mice.

**Cystatin C Amyloid Is Associated with L68Q Epididymal Spermatozoa**—Having shown there is increased cystatin C amyloid in the L68Q epididymal fluid, experiments were next carried out to determine if the cystatin C amyloids were associated with the sperm surface and membrane. Cauda epididymal fluid from WT and L68Q mice. As shown in Fig. 6, fibrillar structures characteristic of amyloid were present in both caput and cauda samples from WT and L68Q mice. However, pellet 4 from L68Q fluid contained more fibrils as well as large, dense structures that appeared to represent bundles of fibrils compared with those present in WT pellet 4 (Fig. 6). Together these studies show that there is more cystatin C amyloid as well as

**FIGURE 5. Identification of cystatin C amyloid in the mouse epididymal fluid.** A, analysis of cystatin C (cc) immunoreactivity followed by thioflavin S staining was performed on epididymal luminal fluid pellet 4 isolated from the caput and corpus/cauda from WT and L68Q mice. NRS, normal rabbit serum. B, immunofluorescence analysis of amyloid in epididymal pellet 4 using the conformation-dependent OC antibody. C, Western blot analysis of cystatin C immunoreactivity in Start (before incubation with PAD ligand) and PAD-bound samples treated with (+) and without (−) DMSO before PAD binding. Epididymal pellet 4 was isolated from the caput and corpus/cauda epididymis from WT and L68Q mice. Proteins were resuspended in PBS, and 2.5 μg of starting material was loaded for each sample on the SDS-PAGE. Equal micrograms of protein (15–20 μg) from each sample were then incubated with PAD ligand with and without exposure to DMSO, and eluted proteins loaded on the SDS-PAGE. D, immunofluorescence analysis of cystatin C (AlexaFluor-594, red fluorescence) and Thioflavin S (yellow-green fluorescence) in epididymal pellet 4 after various times (min) of exposure to 90% DMSO. A, B, D, Bar, 10 μm. For all immunostaining or thioflavin S staining, images were captured with the same exposure times. If brightness was adjusted, equivalent adjustments were also made to controls.
mal spermatozoa were isolated from WT and L68Q epididymides and washed gently to remove loosely associated luminal fluid proteins. The washed spermatozoa were then incubated in low salt buffer to extract surface associated proteins followed by incubation in 1% Triton X-100 to extract membrane-associated proteins. The extracted proteins were then incubated with the PAD ligand followed by Western blot analysis to examine the levels of cystatin C amyloids. In the L68Q spermatozoa there were increased levels of cystatin C amyloid in the low salt and in the Triton X-100 extracted fractions that bound to the PAD ligand compared with that in WT spermatozoa (Fig. 7). These studies demonstrate there is more cystatin C and cystatin C amyloid associated with the surface and membranes of L68Q cauda epididymal spermatozoa compared with control spermatozoa.

Coincubation of WT Spermatozoa with WT and L68Q Luminal Fluid—To directly test if the cauda epididymal luminal fluid from the L68Q mice was cytotoxic to spermatozoa, washed WT spermatozoa were incubated with epididymal luminal fluid isolated from WT or L68Q mice and sperm viability, and motility was determined. Using the MTT assay to examine cell viability, WT spermatozoa incubated in L68Q luminal fluid showed no difference in viability when incubated with either of the two luminal fluid preparations. However, by 60 min the effects of L68Q luminal fluid on the spermatozoa could be detected by a significant decrease in the viability of the WT spermatozoa (p < 0.05). Furthermore, when the motility of WT spermatozoa was examined after 60 min of incubation in the luminal fluid, the spermatozoa incubated with the L68Q luminal fluid showed significantly less progressively motile sperm than those incubated with WT luminal fluid (p < 0.05) (Fig. 8B).

To deplete insoluble amyloids from the luminal fluid, samples from WT and L68Q mice were centrifuged at 250,000 × g, and the resulting supernatant was incubated with WT spermatozoa and motility examined. As shown in Fig. 8C, the depleted L68Q luminal fluid (L68Q SUP) was not harmful for sperm motility as WT spermatozoa showed similar levels of motility as WT spermatozoa incubated with depleted luminal fluid generated from WT mice (WT SUP). Interestingly, WT spermatozoa incubated with depleted luminal fluid from WT or L68Q mice showed a higher percentage of cells that were progressively motile than spermatozoa incubated in nondepleted luminal fluid. The increase in sperm motility followed by the removal of insoluble amyloid may reflect a functional role for the structures in maintaining cauda spermatozoa in an immobilized state in the epididymal lumen (47). Because pellet fractions were difficult to resuspend to homogeneity, they were not incubated with spermatozoa to examine effects on motility. PAD pulldown of the total luminal fluid and supernatant and pellet fractions after exposure to DMSO confirmed that the 250,000 × g centrifugation depleted the cystatin C amyloid from the luminal fluid as all the PAD-bound cystatin C amyloid was in the pellet with none remaining in the supernatant fraction (Fig. 8D). These studies show that the cauda luminal fluid from the L68Q mice is distinct from that of WT mice and contains material that is damaging for sperm function.

**DISCUSSION**

A single point mutation (L68Q) in the human cystatin C gene results in a highly unstable and highly amyloidogenic cystatin C variant. The L68Q cystatin C readily forms amyloid at body temperature as compared with the more stable wild type cystatin C protein that can also form amyloid but to a much lesser degree under normal biological conditions. Because of its highly amy-
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loidalogenic properties, patients with this mutation (HCCAA, human cystatin C amyloid angiopathy) rapidly develop L68Q cystatin C amyloid deposits in the cerebral arteries such that detrimental effects including stroke, dementia, and death can often occur as early as in their 30s (36). Although L68Q cystatin C deposits are also present in organs outside of the brain including the testis, less is known regarding the pathophysiology of these inclusions since the primary cause of death in affected individuals is due to cerebral hemorrhage (36, 37).

Using a mouse model for the study of HCCAA, our studies presented herein demonstrate that L68Q amyloids develop within the reproductive tract and can have detrimental effects on fertility. Specifically, our studies show that heterozygous mice expressing human L68Q cystatin C are unable to generate offspring. Examination of the L68Q spermatozoa showed that sperm viability was determined by adding MTT at time 0 and examining accumulation of MTT crystals after 15, 30, 60, 120, and 180 min. Values represent the mean ± S.E. of 4 experiments. *, p ≤ 0.05. B, the percentage of spermatozoa that showed progressive motility was determined by visual counting of spermatozoa after 60, 90, and 120 min incubation. Values represent the mean ± S.E. of five experiments. *, p ≤ 0.05. C, luminal fluid from WT and L68Q mouse epididymides was depleted of insoluble material by centrifugation at 250,000 × g, and equal µg of the resulting supernatant were incubated with WT spermatozoa, and the percentage of spermatozoa with progressive motility was determined. Values represent the mean ± S.E. of three experiments. *, p ≤ 0.05. D, luminal fluid (total) and luminal fluid that was centrifuged at 250,000 × g to yield a supernatant (SUP) and pellet were treated with DMSO before PAD pulldown to isolate amyloids followed by Western blot analysis using the cystatin C antibody. Start, 5 µg total luminal fluid before incubation with PAD ligand. Equal µg (40 µg) of protein were used for PAD pulldown assays.

Further examination of the L68Q spermatozoa showed that when diluted into aqueous buffers, the viability was reduced compared with that of WT spermatozoa and that large agglutinated clumps of spermatozoa were present. Taken together these studies suggested that in the L68Q mice components in the epididymal luminal fluid surrounding the spermatozoa may bind to the sperm surface causing agglutination and damaging cell membranes such that viability was reduced that ultimately affected sperm motility and fertility. Indeed, when WT spermatozoa were incubated in epididymal fluid from L68Q mice they exhibited a time-dependent reduction in cell viability and motility. The recapitulation of the phenotype of L68Q spermatozoa in WT spermatozoa by the addition of luminal fluid from L68Q mice suggests a causative role for the luminal fluid proteins in the generation of the phenotype.

Our studies further suggest that the presence of the highly amyloidogenic L68Q cystatin C in the epididymal fluid contributed to the infertility phenotype of the L68Q spermatozoa. Increased amounts of cystatin C amyloid were detected in the epididymal fluid surrounding the spermatozoa, and increased amounts of cystatin C amyloid were associated with the sperm surface and membranes. Also, after centrifugation to deplete insoluble material from the luminal fluid, the depleted luminal fluid from L68Q mice was no longer damaging to spermatozoa, and motility was similar to spermatozoa incubated in WT fluid. PAD pulldown assays showed that the depleted luminal fluid no longer contained cystatin C amyloids, suggesting a role for the
amyloids in affecting sperm function. Our studies also showed that the L68Q mice contained different types of amyloid structures in the epididymal fluid compared with that in the WT mice. Although we were unable to determine that in particular human L68Q cystatin C amyloid rather than increased amounts of mouse cystatin C amyloid or a combination of these two was the causative factor, our studies do show that the presence of the human L68Q cystatin C amyloid changed the epididymal luminal environment such that it was incompatible with normal sperm function.

Other transgenic mouse models overexpressing human L68Q cystatin C have been generated; however, none showed fertility defects. This may reflect the different promoters that were used to direct cystatin C expression as the neuron-specific Thy1 promoter used by Kaeser et al. (20) would likely not result in human cystatin C expression in the mouse epididymis. Similarly, the use of the human promoter (18, 49) may result in different levels or patterns of expression of human cystatin C in the epididymis as compared with the mouse cystatin C promoter used in our studies. L68Q human cystatin C expression was not examined in the epididymis in any of these other L68Q mouse models.

Although amyloids play a causative role in a number of invariable neurodegenerative diseases including Alzheimer disease, amyloid structures also have been shown to carry out biological roles in the absence of pathology (51, 52). We have previously established that cystatin (CRES) amyloids are a normal component of the mouse epididymal lumen (41). Although studies are in progress to determine their roles in the epididymis, these functional amyloids are likely formed under controlled conditions such that pathologies do not result as generally occurs during amyloidogenesis. Our studies suggest that environments containing functional amyloids, however, can become pathological if the delicate balance between monomer and amyloid is disturbed possibly due to quality control mechanisms that become overwhelmed or perhaps as a result of an alteration of the functional amyloid structure itself by association/integration of additional amyloids. In our studies the epididymal fluid from the L68Q mice contained more cystatin C protein in the film-like amyloid that is a component of the normal luminal environment as well as additional and distinct stable DMSO resistant cystatin C amyloid structures, suggesting that both mechanisms may have contributed to the fertility defect in the L68Q mouse model.

Amyloids are present in human semen and have been implicated in facilitating HIV infection (53, 54); however, whether amyloids can affect human sperm function and contribute to male factor infertility has not been established. It is of interest, however, that ALS patients show reduced fecundity (48), and individuals with hereditary apolipoprotein A-I amyloidosis exhibit infertility and hypergonadotropic hypogonadism (50). Our studies examining the L68Q mouse model for the human disease HCCAAMS suggest that pathological amyloids can indeed play a role in male infertility by affecting sperm function. Together these studies suggest that the presence of amyloids be considered as a potential causative factor in cases of idiopathic male infertility.

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