Brief Report

Title:
Targeted mutation of zebrafish fga models human congenital afibrinogenemia

Short title:
Afibrinogenemia in zebrafish

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Scientific category: THROMBOSIS AND HEMOSTASIS
Key points

- Targeted mutation of a zebrafish fibrinogen gene leads to a bleeding phenotype, analogous to human congenital afibrinogenemia
- This first heritable coagulopathy model validates the use of zebrafish for thrombosis and hemostasis research

Abstract

Mutations in the human fibrinogen genes can lead to the absence of circulating fibrinogen and cause congenital afibrinogenemia. This rare bleeding disorder is associated with a variable phenotype, which may be influenced by environment and genotype. Here we present a zebrafish model of afibrinogenemia. We introduced targeted mutations into the zebrafish fga gene using zinc finger nuclease technology. Animals carrying three distinct frameshift mutations in fga were raised and bred to produce homozygous mutants. Using a panel of anti-zebrafish fibrinogen antibodies, fibrinogen was undetectable in plasma preparations from homozygous mutant fish. We observed hemorrhaging in fga mutants and reduced survival compared to control animals. This model will now serve in the search for afibrinogenemia modifying genes or agents and, to our knowledge, is the first transmissible zebrafish model of a defined human bleeding disorder.
Afibrinogenemia is defined by undetectable circulatory fibrinogen which can lead to a variety of bleeding and thrombotic complications for affected individuals. The soluble precursor to fibrin, fibrinogen is a conserved hexameric protein with its three polypeptide chains, Aα, Bβ, and γ, encoded by three genes; FGA, FGB and FGG in humans. Minor isoforms of Aα (Aα-E) and γ (γ') arise by differential mRNA splicing. Mutations in one of the fibrinogen genes can cause afibrinogenemia, but causal mutations have been found predominantly in FGA. Afibrinogenemia is rare, affecting about 1 in 10^6 individuals, and can be treated by replacement therapy. However, as with other heritable bleeding disorders, the variable phenotype of afibrinogenemia is not fully understood and an experimental system where genetic and environmental influences on phenotype could be assessed would be a useful research tool.

While targeted disruption of Fga in mice clearly leads to an afibrinogenemia-like phenotype we sought to produce a zebrafish model of afibrinogenemia. The zebrafish (D. rerio) is used as a model vertebrate in developmental biology and for modeling human disease. Coagulation factors have been described in zebrafish, their genes are identifiable in the zebrafish genome, and hemostasis assays have been adapted to the model. With large numbers of offspring and rapid external early development the zebrafish is compatible with genetic or chemical screening approaches, such as searching for phenotypic modifier genes or therapeutic agents. In addition, we anticipated that female zebrafish lacking circulating fibrinogen may not incur the lethality seen in gestating afibrinogenemic mice.

We previously characterized the zebrafish fibrinogen genes and their expression during development. Furthermore, a transitory model of low fibrinogen levels in zebrafish larvae was recently reported using antisense morpholino oligonucleotides targeting fibrinogen mRNAs. However, no evidence for lowered fibrinogen protein levels was provided. A zebrafish model of a congenital human bleeding disorder, with a stable genetic change linked to a relevant phenotype, has not been described. Here we present a zebrafish model of congenital afibrinogenemia. We engineered mutations into the zebrafish fga gene that lead to afibrinogenemic fish with a bleeding phenotype.
Methods

Zebrafish

AB strain zebrafish were maintained at 28 °C, pH 7.5 and 500 µS conductivity. Experimentation was authorized by the local veterinary authority.

fga mutants

Plasmids encoding zinc finger nucleases (ZFN) designed to target the \( fga \) gene, were purchased from Sigma-Aldrich. In vitro transcribed mRNA encoding each half of the dimeric zinc finger nuclease were microinjected into early zebrafish embryos. Surviving fish were raised to reproductive age and screened for transmission of mutated \( fga \) by PCR-genotyping embryos from natural crosses. The ZFN used targets a sequence overlapping a \( BceAI \) restriction site in \( fga \) exon 2 (see Figure 1A). This enabled identification of mutated \( fga \) by \( BceAI \) digestion of PCR products. Oligonucleotides used were forward: 5’-AATGGCCTATGTTGGCAGAC-3’ and reverse: 5’-CAGTGGTTATCAGCTGACAG-3’. \( BceAI \)-resistant PCR products were sequenced and two fish transmitting predicted null alleles were crossed with wild type fish and progeny raised and screened for heterozygosity. Heterozygotes were in-crossed. We identified heterozygous animals from this subsequent generation, for each mutation, because one line had transmitted two mutations, presumably from separate ZFN-directed editing events in the founder animal. Heterozygotes for each of the three mutations were confirmed by sequencing cloned genotyping PCR products (pCRII TOPO, Life technologies). Subsequent in-crosses gave wild type, heterozygous and homozygous fish. Zebrafish experimentation was authorized by the local veterinary authority.

Detection of larval bleeding

Bleeding was assessed in three or five day-old larvae using o-dianisidine staining, essentially as described previously, with post-staining fixation.

Plasma collection and western blotting

Plasma was prepared from adult fish using a described protocol as a guide. Blood was collected into 300 µl of heparin solution (420 IU/ml) after tail sectioning of anaesthetized fish. Samples of dilute plasma were subjected to western blotting. Antibodies were prepared by immunization of rabbits with peptides found in each of the zebrafish fibrinogen chains (Covalab, France). Peptide sequences, their
positions in each fibrinogen chain and associated UniProtKB accession numbers were as follows: \( \alpha \) IEHGFKAQDTCQTEW (amino acids 33-48) E9QCD1, \( \beta \) GKAVQKEEKPEESGCC (amino acids 75-90) Q6NYE1, \( \gamma \) (amino acids 50-65): DYLQRYKPDMDKLDD (50-65) Q7ZVG7. Anti-zebrafish ceruloplasmin antibodies were purchased (Eurogentec, Belgium).

**RNA isolation and RT-PCR**

RNA was isolated from adult liver samples using Trizol reagent (Life technologies) and reverse transcription-PCR performed as described previously, using unchanged oligonucleotides.\(^{10}\)
Results and Discussion

Generating fga mutant zebrafish

The fibrinogen hexamer is assembled from three polypeptide chains; severe truncation of one chain is predicted to cause the absence of circulating fibrinogen. Exon 2 of the zebrafish fga gene, encoding fibrinogen Aα (and Aα-E) was targeted for mutation using ZFN technology (Figure 1A). Fish subjected to the ZFN were raised and animals transmitting mutations predicted to give null alleles were out-crossed. Three mutation-transmitting lines were maintained (Figure 1A). The mRNA resulting from each modification is predicted to have an open reading frame shift and encode a non-functional truncated fibrinogen Aα (see Supplemental data). A generation from mating heterozygotes of each mutant line was genotyped aged 4-5 months; in total 236 fish were analyzed (Figure 2A).

fga mutants are afibrinogenemic

We collected blood samples from wild type (+/+), heterozygous (+/-) and homozygous (-/-) animals from each mutant line and subjected diluted plasma to western blotting using antibodies that recognize zebrafish fibrinogen Aα, Bβ and γ chains and ceruloplasmin as a control plasma protein. No fibrinogen was detected in the blood of homozygous mutants (Figure 1B), demonstrating that the mutations described render zebrafish afibrinogenemic. We dissected liver tissue to analyze fibrinogen mRNA expression by RT-PCR. Expression of fga is reduced in -/- animals, presumably by nonsense-mediated decay (Figure 1C). DNA sequencing of the fga cDNA confirmed the presence of wild type and mutated transcripts in +/+ and -/- animals for each mutant line (not shown).

Effects on survival

Compared to a Mendelian ratio of 0.25 +/+: 0.5 +/-: 0.25 -/- genotypes, -/- fish were under-represented for mutations 1 and 2 in the live genotyped fish (Figure 2A). 13/104 fish were mutation 1 homozygous mutants, compared to 36/104 wild types. 10/73 fish were mutation 2 homozygous mutants, compared to 25/73 wild types. This implies survival of only ~40% of the homozygotes from heterozygous in-crossings. Concordant with this data, homozygous mutants were over-represented in dead fish recovered from the mutation 1 and 2 groups (see Supplemental data). This demonstrates reduced survival with two of the homozygous fga mutations, although the effect was not seen with mutation 3.
in the generation described. Analysis of further generations will determine whether mutation 3 confers modified survival compared to mutations 1 and 2.

**A bleeding phenotype**

Criteria for a model of human afibrinogenemia include the absence of circulating fibrinogen (Figure 1B) and a variable bleeding phenotype. We sporadically observed bleeding in fish from the lines described and suspected catastrophic bleeding events to explain fatalities of mutant fish. Figure 2B-D show examples of unprompted bleeding found in adult mutant fish. Images were taken prior to genotyping. These bleeding events were variable; cephalic (Figure 2B and 2D), ocular (Figure 2B), and ventral hemorrhaging (Figure 2C) are represented. Such incidents were observed in less than 10% of the expected number of mutation 1 and 2 homozygotes. However, if lethal bleeding events explain the under-representation of the -/- genotype in live adult fish, compared to +/- siblings, such events occurred in approximately 60% of mutation 1 and 2 homozygotes. We did not detect severe bleeding in live mutation 3 homozygous mutants in the generation described, in accordance with our survival data.

We looked for bleeding in 3 and 5 day-old mutant larvae by staining with o-dianisidine to detect heme. We observed pools of apparent blood extravasation in 3 day-old mutant larvae, but these events were rare and also seen in wild types (see Supplemental data for images and bleeding frequencies). Either larval mutants do not suffer from additional bleeding events compared to wild types, or with the numbers of larvae analyzed we have not detected it in the current study. Our data imply that severe bleeding as a phenotypic end-point for further study will require large numbers of adult or larval afibrinogenemic zebrafish.

In summary, we describe zebrafish lacking circulating fibrinogen, presenting a bleeding phenotype and reduced survival. Unprompted bleeding was rare, but the phenotypic similarity with human congenital afibrinogenemia validates the use of the zebrafish to search for modifier genes or agents that by functional conservation could affect the human disease. Our findings also infer that the zebrafish can be used as a model for other congenital bleeding disorders.
Acknowledgements

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Authorship Contributions

RJF designed the study, performed zebrafish experiments and wrote the paper, CDS performed experiments and MNA directed the study and wrote the paper.

Disclosure of Conflicts of Interest

The authors have no conflicts of interest to declare.
References


**Figure Legends**

**Figure 1 Generation of afibrinogenemic zebrafish**

Mutations were introduced in exon 2 of the zebrafish *fga* gene by ZFN-mediated editing. (A) The mutations in zebrafish lines described are represented. The exon-intron composition of the two zebrafish *fga* transcripts, encoding fibrinogen Aα (upper) and Aα-E (lower) are sketched at the top of the panel. Coding regions are broad, non-coding parts of the transcripts and introns are narrow. The arrow represents the transcriptional orientation. The mutated region of exon 2 is zoomed showing the local single letter amino acid sequence in the wild type Aα or Aα-E, above the nucleotide sequence within exon 2 for wild type and mutant zebrafish described. The underlined nucleotides in the wild type sequence are a BceAI recognition sequence, disrupted in the mutants described and used for genotyping. The blue letters represent the position of the designed interaction site for the ZFN used; only one DNA strand is shown for clarity. Hyphens represent deleted nucleotides. Mutation 1 is a 10 nucleotide deletion and 3 nucleotide insertion, mutation 2 a 14 nucleotide deletion and mutation 3 a 7 nucleotide deletion. (B) Reduced (R) plasma preparations from wild type (+/+), heterozygous (+/-) and homozygous (-/-) mutant fish were subjected to western blotting using antibodies to zebrafish fibrinogen Aα, Bβ and γ chains and ceruloplasmin (CP) as a loading control for each blot. A second Aα blot was made with non-reduced samples to detect the fibrinogen hexamer (NonR). The additional high molecular weight band in the mutation 1 wild type (+/+ non-reduced Aα blot is thought to be a small amount of fibrin polymer that may have formed rapidly upon tail sectioning, prior to immersion in heparin solution. Fibrinogen was not detected in any of the homozygous mutant fish plasma samples (-/-). The additional higher molecular weight anti-Aα reactive band in the mutation 3 +/- and +/+ samples, marked with an asterisk, is thought to be the Aα-E isoform. Its absence in mutation 1 and 2 +/- and +/+ samples is due to a polymorphism seen on the non-mutated *fga* allele in these lines that is predicted to truncate the Aα-E isoform (see Supplemental data). (C) RT-PCR products run on agarose gels are shown for *fga*, *fgb*, *fgg* and ceruloplasmin (*cp*) cDNA in wild type (+/+), heterozygous (+/-) and homozygous mutant fish (-/-) liver samples for each zebrafish line. The - or + under each lane denotes PCR reactions made on cDNA synthesis reactions without (-) or with (+) reverse transcriptase.
**Figure 2 Survival and bleeding of afibrinogenemic zebrafish**

A generation of adult zebrafish from mating of heterozygous animals from each mutant line was genotyped for exon 2 of the \textit{fga} gene aged 4 to 5 months. The percentage of fish for each \textit{fga} genotype, wild type (+/+) , heterozygous (+/-) and homozygous mutant fish (-/-) is represented in A.

The total number of live animals genotyped for each line in this generation is shown to the right of each bar. Compared to an expected Mendelian ratio, homozygous mutant fish (-/-) are under-represented and wild-type (+/+) fish over-represented in animals from heterozygous mutation 1 and 2 crosses. In B to D, unprompted bleeding events from mutation 1 and 2 fish are represented. Black and white arrows highlight sites of bleeding. B shows a cephalic and ocular bleed with right side (left image) and dorsal views (right image). C shows a ventral hemorrhage with left side (left image) and ventral views (right image) and D shows a dorsal view of a cephalic bleed (left image) with a non-bleeding fish for comparison (right image). Scale bars are given in each image. Images were acquired using a Leica MZ16FA stereomicroscope equipped with a DFC420 digital camera and the Leica Application Suite software V4.2. A 1.0x Planapochromatic objective was used. The zoom magnification was between 0.71 and 1.48 with a visual magnification between 7.1 and 14.8. The numerical aperture was 0.020 (B and C) or 0.040 (D). Color was set to a saturation setting of 1.7 to 2.0 and gamma at 0.99. Images were taken at room temperature.
Figure 1
Figure 2
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