Manipulating the quality control pathway in transfected cells: low temperature allows rescue of secretion-defective fibrinogen mutants

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Abstract

BACKGROUND: Congenital afibrinogenemia is characterized by the absence of fibrinogen, a hexamer composed of two copies of three polypeptides, Aalpha, Bbeta and gamma. The disease is caused by mutations in one of the three fibrinogen-encoding genes, FGA, FGB and FGG. Among these, several mutations have been reported to specifically impair fibrinogen secretion. We previously showed that secretion-defective fibrinogen mutants are retained in a pre-Golgi compartment and demonstrated the importance of the homologous betaC and gammaC domains in secretion. Here our aim was to restore the secretion of these mutants and study the properties of the rescued mutant molecules. DESIGN AND METHODS: COS-7 cells were transfected and incubated with chemical chaperones or at low temperature. Clotting assays and plasmin digestion studies were performed to characterize secreted fibrinogen molecules. RESULTS: The secretion defect of two missense mutants but not that of late-truncating mutants could be partially corrected by incubating cells at 27 degrees C. By contrast, exposure of cells to chemical chaperones i.e. 4-phenylbutyrate, dimethyl [...]
Manipulating the quality control pathway in transfected cells: low temperature allows rescue of secretion-defective fibrinogen mutants

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ABSTRACT

Background
Congenital afibrinogenemia is characterized by the absence of fibrinogen, a hexamer composed of two copies of three polypeptides, Aα, Bβ, and γ. The disease is caused by mutations in one of the three fibrinogen-encoding genes, FGA, FGB, and FGG. Among these, several mutations have been reported to specifically impair fibrinogen secretion. We previously showed that secretion-defective fibrinogen mutants are retained in a pre-Golgi compartment and demonstrated the importance of the homologous βC and γC domains in secretion. Here our aim was to restore the secretion of these mutants and study the properties of the rescued mutant molecules.

Design and Methods
COS-7 cells were transfected and incubated with chemical chaperones or at low temperature. Clotting assays and plasmin digestion studies were performed to characterize secreted fibrinogen molecules.

Results
The secretion defect of two missense mutants but not that of late-truncating mutants could be partially corrected by incubating cells at 27°C. By contrast, exposure of cells to chemical chaperones, i.e., 4-phenylbutyrate, dimethyl sulfoxide or trimethylamine N-oxide had no effect. The mutants rescued at 27°C were incorporated into fibrin clots and formed factor XIII-mediated γ-γ dimers in contrast to the dysfibrinogenemia Vlissingen/Frankfurt IV mutant, a negative control for these assays. However, plasmin digestion analyses revealed aberrant patterns for the mutants compared to normal fibrinogen.

Conclusions
Low temperature can restore the secretion of a subset of mutant fibrinogen molecules demonstrating that therapeutic manipulation of the quality control pathway is feasible for afibrinogenemia even though functional assays suggested a non-native conformation for the mutant molecules analyzed.

Key words: fibrinogen, afibrinogenemia, secretion, chemical chaperones, protein quality control, fibrinogen-related domain

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Introduction

Congenital afibrinogenemia (OMIM #202400) is a rare bleeding disorder characterized by the complete absence of fibrinogen, the precursor of fibrin, which is the major protein component of blood clots. Affected patients suffer from various hemorrhagic manifestations, bleeding from the umbilical cord often being the first sign of the disorder. Other symptoms manifest with variable severity and include oral, musculoskeletal and intracranial bleeding, hemarthrosis, epistaxis, menorrhagia and recurrent early abortions. Patients may, however, also experience thrombotic events. Current therapy for afibrinogenemia is based on fibrinogen replacement, usually in the form of cryoprecipitate or fibrinogen concentrates. Afibrinogenemia is caused by mutations, in homozygosity or compound heterozygosity, in one of the three fibrinogen-encoding genes, FGA, FGB and FGG, clustered on human chromosome 4 (4q32.1). Mutations in these three genes are responsible for almost all cases of congenital afibrinogenemia, with the exception of some sporadic cases and isolated families.
(p.N319_D320del) mutation was introduced in the tricistronic fibrinogen-containing plasmid with the forward oligonucleotides as follows (introduced mutations are underlined): 5'-GATGGTGTAGTATAAGATGAATTGGAAAGGGG-3' for FGB p.W467X (p.W437X), 5'-GCAGCCAATCCAAACTGCAGATACTCTGGGGG-3' for FGB p.G444S (p.G414S), 5'-GCACAAACAGATTGGAGATGAATTGGAAAGGGG-3' for FGG p.W253C (p.W227C), and 5'-CCTGGGAGCAATGACTGGGGG-3' for FGG p.N345_D346del (p.N319_D320del) (6-nucleotide deletion between the two underlined nucleotides). The resulting constructs were checked by sequencing all three fibrinogen cDNA.

Higher fibrinogen expression was obtained with single transfection of tricistronic constructs in comparison with triple co-transfections, but with similar results as regards protein assembly and secretion (data not shown). In this study single transfections with tricistronic constructs were performed for expression of the γ p.N345_D346del (p.N319_D320del) mutant and all clotting and plasmin digestion assays while triple co-transfections were performed for cell treatment with chemical chaperones or low temperature incubation (see online supplement for further details).

**Results**

**Rescue of secretion-defective mutants**

To investigate whether the secretion of the fibrinogen FGB p.G444S (p.G414S), FGB p.W467X (p.W437X) and FGG p.W253C (p.W227C) mutants can be rescued, we co-transfected COS-7 cells with empty vectors, the three normal fibrinogen cDNA constructs, or the mutant FGB G444S, FGB W467X, FGG W253C cDNA (residues numbered from the initiator codon) combined with the other two corresponding normal cDNA. Cells were exposed to one of the three common chemical chaperones with concentrations found to be successful in other studies (10 mM 4-phenylbutyrate (4-PBA), 2% dimethylsulfoxide (DMSO), and 100 mM trimethylamine N-oxide (TMAO). Cells were also incubated at a temperature of 27°C. Two days after transfection, cell extracts and culture media were harvested and analyzed by western blotting.

Figure 1 shows that in all conditions wild-type fibrinogen chains were expressed and assembled into hexameric molecules which were secreted into the culture medium. In non-treated cells, the three mutants ββ p.G444S (p.G414S), ββ p.W467X (p.W437X) and γ p.W253C (p.W227C) were
also expressed and incorporated into hexamers (Figure 1A, cell extract), but these were not secreted (Figure 1A, culture medium), as previously shown. Upon exposure of cells to the chemical chaperones 4-PBA, DMSO and TMAO, expression and assembly of the mutants was unaltered (Figure 1B-D, cell extract), but secretion was not restored (Figure 1B-D, culture medium). By contrast, when cells were incubated at 27°C, the two missense mutants i.e. Bβ p.G444S and γp.W253C (p.W227C), but not the nonsense mutant Bβ p.W467X (p.W437X), were secreted, albeit to a lesser extent than wild-type fibrinogen (Figure 2A culture medium). We also investigated whether two secretion-defective mutants in FGB designed for our previous study were secreted at low temperature; interestingly, the secretion of the Bβ p.R485X (p.R455X) mutant was not restored, while that of the Bβ p.R485A+P486X (p.R455A+P456X) mutant, differing from the former only by an additional alanine in the C-terminus, was partially restored (Figure 2B, culture medium).

**Secretion of the dysfibrinogenemia Vlissingen/Frankfurt IV/Otsu mutant**

As a negative control for the functional analyses that we wished to perform on the rescued mutant molecules i.e. polymerization and conformation, we used the dysfibrinogenemia γ mutant Vlissingen/Frankfurt IV/Otsu. This mutant was identified in heterozygosity in two unrelated families and is characterized by the deletion of two residues, p.N345_D346del (p.N319_D320del), in the γ chain. Studies with purified patient plasma comprising a mixture of normal and mutant fibrinogen showed impaired calcium binding, fibrin polymerization, factor XIII (FXIII)-catalyzed cross-linking and platelet adhesion. Other studies with pure recombinant γ p.N345_D346del (p.N319_D320del) fibrinogen expressed from CHO cells confirmed these observations, suggesting that the two-residue deletion affects the structural integrity of the C-terminal domain of γ chains. Here we expressed the γ mutant Vlissingen/Frankfurt IV/Otsu. This mutant was identified in heterozygosity in two unrelated families and is characterized by the deletion of two residues, p.N345_D346del (p.N319_D320del), in the γ chain. Studies with purified patient plasma comprising a mixture of normal and mutant fibrinogen showed impaired calcium binding, fibrin polymerization, factor XIII (FXIII)-catalyzed cross-linking and platelet adhesion. Other studies with pure recombinant γ p.N345_D346del (p.N319_D320del) fibrinogen expressed from CHO cells confirmed these observations, suggesting that the two-residue deletion affects the structural integrity of the C-terminal domain of γ chains.

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**Figure 2. Rescue at 27°C of secretion-defective fibrinogen mutants, either (A) naturally occurring in patients, or (B) experimentally designed.** Samples were resolved on 9% or 7% SDS-PAGE under reducing or non-reducing conditions, respectively, and immunoblotted with anti-fibrinogen antibodies. The positions of Asp, Bβ, γ chains and hexamers are shown. Open arrowheads indicate fibrinogen intermediates, presumably [AβBγ] half-molecules and [Aγγ] complexes. Loading was controlled by immunoblotting using anti-actin antibodies. Fg: purified human plasma fibrinogen. An asterisk indicates truncated Bβ p.W467X (p.W437X) chains.

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**Figure 3. Expression, assembly and secretion of the γ p.N345_D346del (p.N319_D320del) fibrinogen mutant.** COS-7 cells were transfected with empty vectors (γ, normal wt) or γ p.N345_D346del (p.N319_D320del) mutant fibrinogen construct. Proteins of cell lysates and media were harvested and separated on 9% or 7% SDS-PAGE under reducing or non-reducing conditions. Immunoblots were carried out with anti-fibrinogen antibodies. Loading was controlled with anti-actin antibodies. The positions of Asp, Bβ, γ chains and hexamers are shown. The open arrowhead indicates probable [AβBγ] half-molecules.
The rescued mutants can be incorporated into a clot and form \( \gamma\gamma \) dimers

We wished to determine whether the two missense fibrinogen mutants \( \beta\beta\) p.G444S (p.G414S) and \( \gamma\gamma\) p.W253C (p.W227C) that are secreted at low temperature might be functional i.e. whether they are able to incorporate into a fibrin clot and form cross-links. To address this question, standard clinical tests (e.g. the Clauss method) or \textit{in vitro} assays\textsuperscript{26,31} which rely on high levels of secreted fibrinogen, cannot be applied to our transfected cell model, where we estimate that only approximately 50 ng/mL of recombinant fibrinogen is secreted into the culture medium. We, therefore, adapted a protocol that measures the incorporation of small amounts of radiolabeled secreted fibrinogen into a clot formed with normal plasma.\textsuperscript{26,27} Normal or mutant recombinant fibrinogen was expressed in transfected COS-7 cells at 27\(^\circ\)C, labeled with \([35\text{S}]\)-methionine/cysteine and incubated at 27\(^\circ\)C to allow secretion of the fibrinogen mutants \( \beta\beta\) and \( \gamma\gamma\) secreted at low temperature might be functional. Similarly, both temperature-sensitive \( \beta\beta\) p.G444S (p.G414S) and \( \gamma\gamma\) p.W253C (p.W227C) mutants could incorporate into the clot and undergo covalent FXIII-catalyzed cross-linking to form \( \gamma\gamma \) dimers (Figure 4B). While the intensity of their corresponding bands was lower than for normal fibrinogen this was not because of lesser efficiency of incorporation into clots, since no fibrinogen was detectable in the supernatant (Figure 4C). This suggests that the COS-7 cell system allows expression and secretion of recombinant fibrinogen proteins that are functional. The rescued fibrinogen mutants show aberrant plasmin digestion profiles

Plasmin sequentially cleaves fibrinogen into four major groups of fragments named X, Y, D and E,\textsuperscript{32,33} a cleavage which depends on the overall three-dimensional structure of fibrinogen.\textsuperscript{44} We analyzed the kinetics of plasmin digests of wild-type, \( \beta\beta\) p.G444S (p.G414S), \( \gamma\gamma\) p.W253C
(p.W227C) or γ p.N345_D346del (p.N319_D320del) mutant fibrinogen molecules secreted by COS-7 cells at permissive temperature. As a control, human purified plasma fibrinogen was digested in parallel. Plasmin digestions were performed at 37°C (Figure 5) or at 27°C (data not shown) with similar results. Western blots in non-reducing conditions (Figure 5A) showed a standard pattern of plasmin digests for purified fibrinogen, with the progressive degradation of full hexamers (t=0) into fragments X, Y, D (detected in our assays from 5 min of incubation with plasmin) and E (from 30 min). After 30 minutes, fragments D and E were predominant. Plasmin digestion of wild-type fibrinogen secreted by COS-7 was indistinguishable from that of plasma fibrinogen, demonstrating that recombinant fibrinogen produced by COS-7 cells has the same susceptibility to plasmin digestion as plasma fibrinogen. The γ p.N345_D346del (p.N319_D320del) mutant showed similar degradation products at 5 minutes of plasmin incubation while after 30 minutes additional bands below fragment D were detected and at 6 hours, partial degradation of fragments D1 into D2 and D3 was clearly observed (Figure 5A) as previously reported. As before, the signals obtained for the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants were weaker because of the smaller amount of molecules secreted at 27°C compared to wild-type or γ p.N345_D346del (p.N319_D320del) mutants. The Bβ G444S mutant showed aberrant degradation products after 5 minutes, migrating at around 70-75 kDa (Figure 5A). The γ p.W253C (p.W227C) mutant had a normal digestion pattern up to 30 minutes of incubation but at 6 hours, one aberrant doublet at approximately 60-65 kDa predominated.

Calcium and the GPRP peptide are known to protect the degradation of fibrinogen fragments D1 into D2 and D3. Assessing the protection from plasmin degradation with one or the other compound indicates whether fibrinogen molecules can bind calcium or have functional “a” polymerization sites, respectively. Indeed, the GPRP peptide mimics the N-terminal “A” site of α chains which are exposed after cleavage of fibrinopeptide A by thrombin. The “A” site interacts with the “a” site present in the outer γD domain of another molecule, allowing the alignment of fibrin monomers into two-stranded, half-staggered protofibrils.

Normal recombinant fibrinogen secreted by COS-7 cells was protected to the same extent as plasma fibrinogen while the γ p.N345_D346del (p.N319_D320del) mutant showed reduced protection against plasmin digestion in

**Figure 5.** Plasmin digestion of fibrinogen. (A) Kinetics of plasmin digestion at 37°C of plasma fibrinogen or culture medium from non-transfected (--) or transfected COS-7 cells in the presence of approximately 0.9 mM CaCl₂ (due to the 1.8 mM CaCl₂ in DMEM) (B) Plasmin protection assay of plasma fibrinogen or culture medium from non-transfected (--) or transfected COS-7 cells in the presence of 10 mM EDTA, 5 mM CaCl₂, or 4 mM GPRP+10 mM EDTA. Positions of fragments X, Y, D1, D2, D3 and E are indicated.
the presence of calcium or GPRP, as shown by the increases of D2 and D3 in these conditions (Figure 5B). Smaller unidentified bands were also detected. Surprisingly, when EDTA was added, some protection remained, contrasting with the results for normal plasma and recombinant fibrinogen, as well as previous studies on the γ p.N345_D346del (p.N319_D320del) mutant.24,25 However, partial protection was also observed for plasma fibrinogen of mice heterozygous for the mutation.26 In the case of the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants, a clearly aberrant pattern of plasmin cleavage was observed. No fragments D could be found in either case. Only smaller bands were present suggesting further digestion beyond fragments D, although altered migration of mutant molecules during SDS-PAGE cannot be ruled out.

### Discussion

Chemical chaperones and low growth temperature are often used as a first step to evaluate the feasibility of treatments using pharmacological chaperones for secretion-impaired mutations. In this context, the examples of the CFTR AF508 or α-antitrypsin Z variants responsible for cystic fibrosis and α-antitrypsin deficiency, respectively, are promising.16-18 In our transfected cell model we found that none of the chemical chaperones used (4-PBA, DMSO and TMAO) restored the secretion of the fibrinogen mutants studied. By contrast, low-temperature incubation (27°C) allowed partial secretion of the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants. Low temperature may act kinetically by slowing down the folding process thus allowing a higher amount of mutant proteins to adopt a suitable conformation and reach their final destination.19 Interestingly, in this condition, the two nonsense mutants Bβ p.W467X (p.W437X) and Bβ p.R485X (p.W455X) were not secreted. This suggests a more severe conformational defect for the nonsense mutants than for the missense mutations analyzed here. Indeed, C-terminal deletions of Bβ chains are thought to destabilize the βC domain, allowing solvent access which may induce an aberrant βC conformation that is too severe to be rescued.11 Interestingly, a small alanine residue (Bβ R485A+P456X or R455A+P456X) placed at the C-terminal entry site of the globular domain appears to be sufficient to partially restore secretion at low temperature. Fibrinogen molecules that contain the two temperature-sensitive mutants Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) fibrinogen seem to be at least partially functional since they can enter a clot and form cross-linked γ-γ dimers, implying fibrinopeptide cleavage by thrombin and proper half-staggered alignment in protofibrils for FXIII-catalyzed cross-linking. We cannot exclude that the mutants are only able to associate with normal plasma fibrinogen molecules (provided in excess in our assay) which would not be possible in the case of a patient homozygous for the mutation. However, we were at least able to demonstrate that these mutants differ from the dysfibrinogen γ p.N345_D346del (p.N319_D320del) mutant, which is unable to polymerize or cross-link, either in pure form or in combination with normal plasma fibrinogen.

Previous studies reported a secretion defect associated with the γ p.N345_D346del (p.N319_D320del) mutant we used here as a control. Indeed, the Otsu patient heterozygous for the γ p.N345_D346del (p.N319_D320del) mutation had intermediate plasma fibrinogen levels of 1.7 g/L, just below the normal range and reduced levels of variant γ chains compared to normal ones.25 Furthermore, mice carrying the same mutation had approximately half (heterozygous animals) and one-eighth (homozygous animals) of the plasma fibrinogen levels of wild-type animals.26 In contrast, normal fibrinogen levels and equal amounts of normal and mutant γ chains were found in the plasma of the Vliissingen patient heterozygous for the mutation,27 and no hypodysfibrinogenemia was reported for the other affected members of the family.27 In our in vitro system, the mutant does not seem to have a defect in secretion whereas in a similar study in CHO cells, a slight reduction in secretion was observed.4 This discrepancy may be attributable to a greater permissiveness of COS-7 cells in comparison with CHO cells regarding secretion of mutant fibrinogen molecules, as we had previously found for assembly of truncated γ chains into hexamers.28 In this regard, half-molecules, [ααγ] complexes and free αα or γ chains in the culture medium of COS-7 cells (Figures 1, 2) were also observed in that of hepatoma cell lines such as Hep3B and HepG2 cells, transfected BHK, CHO and COS-1 cells16,27,42 and even in the plasma of afibrinogenemic patients.24,25 It is difficult to determine which cell model reflects the in vitro situation best, since these differences are also found in patients, possibly reflecting polymorphic differences in modifier genes, which remain to be identified. However, the fact that normal recombinant fibrinogen secreted by COS-7 cells is clottable with a susceptibility to plasmin degradation indistinguishable from plasma fibrinogen indicates that the COS-7 model, like the CHO model,27 is suitable for these studies.

In conclusion, we found that lowering the incubation temperature can restore the secretion of mutant fibrinogen molecules in transfected COS-7 cells, demonstrating that therapeutic manipulation of the quality control pathway is feasible for afibrinogenemia even though functional assays suggested a non-native conformation for the mutant molecules analyzed here.

### Authorship and Disclosures

DV designed and performed the research, analyzed the data and wrote the paper; CDS performed research; MNA designed research, analyzed data and wrote the paper. The authors reported no potential conflicts of interest.
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