A novel natural mutation AαPhe98Ile in the fibrinogen coiled-coil affects fibrinogen function

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Summary
The aim of this study was to investigate the structure and function of fibrinogen obtained from a patient with normal coagulation times and idiopathic thrombophilia. This was done by SDS-PAGE and DNA sequence analyses, scanning electron microscopy, fibrinopeptide release, fibrin polymerisation initiated by thrombin and reptilase, fibrinolysis, and platelet aggregometry. A novel heterozygous point mutation in the fibrinogen Aα chain, Phe98 to Ile, was found and designated as fibrinogen Vizovice. The mutation, which is located in the RGDF sequence (Aα 95–98) of the fibrinogen coiled-coil region, significantly affected fibrin clot morphology. Namely, the clot formed by fibrinogen Vizovice contained thinner and curled fibrin fibers with reduced length. Lysis of the clots prepared from Vizovice plasma and isolated fibrinogen were found to be impaired. The lysis rate of Vizovice clots was almost four times slower than the lysis rate of control clots. In the presence of platelet agonists the mutant fibrinogen caused increased platelet aggregation. The data obtained show that natural mutation of Phe98 to Ile in the fibrinogen Aα chain influences lateral aggregation of fibrin protofibrils, fibrinolysis, and platelet aggregation. They also suggest that delayed fibrinolysis, together with the abnormal fibrin network morphology and increased platelet aggregation, may be the direct cause of thrombotic complications in the patient associated with pregnancy loss.

Keywords
Fibrinogen, platelet aggregation, RGD sequence, thrombosis, pregnancy loss

Introduction
Thrombosis may become a serious life-threatening condition which requires medical attention. Although common risk factors for thrombosis are well established, e.g. atherosclerosis, factor V Leiden, or prothrombin mutation G20210A, about 30% of thrombosis cases have unknown aetiology. Fibrinogen is an independent cardiovascular risk factor and it is well known that the circulating level of fibrinogen is associated with the risk of thrombotic diseases, especially if fibrinogen is present in excess or in a variant form. The precise role of fibrinogen in such diseases is still not well understood. The studies of fibrinogen variants enable better understanding of the structure and function of the molecule, especially in pathological processes.

Fibrinogen is a 340 kDa glycoprotein consisting of three pairs of different polypeptide chains, Aα (66 kDa), Bβ (52 kDa), and γ (46 kDa) (1). These chains are encoded by distinct genes (FGA, FGB, and FGG) clustered on chromosome 4 (4q31.3) (2, 3). Fibrinogen chains are synthesised in hepatocytes in the rough endomucos reticulum and, after being properly assembled, fibrinogen is secreted into the blood stream. Fibrinogen consists of two distal D-regions connected to the central E-region by two coiled-coil connectors. Each connector contains helical segments of all three D-regions connected to the central E-region by two coiled-coil connectors. Each connector contains helical segments of all three D-regions connected to the central E-region by two coiled-coil connectors. Each connector contains helical segments of all three D-regions connected to the central E-region by two coiled-coil connectors. Each connector contains helical segments of all three D-regions connected to the central E-region by two coiled-coil connectors. Each connector contains helical segments of all three D-regions connected to the central E-region by two coiled-coil connectors.

In the present study, we report a novel natural point mutation in the fibrinogen Aα chain, Phe98 to Ile, which is located next to
the RGD sequence. Such mutant fibrinogen, designated as fibrinogen Vizovice, is characterised by abnormal fibrin polymerisation, delayed fibrinolysis, and increased platelet aggregation.

Materials and methods

Materials

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified.

Sample collection and preparation

A final volume of 9 ml blood was collected into 1 ml of 3.8% trisodium citrate by venipuncture. Plasma, either from healthy donors or the patient, was prepared by centrifugation for 10 minutes (min) at 1,400×g at 25°C. All individuals tested agreed to this study at the time of blood collection. All samples were obtained in accordance with the Ethical Committee regulations of the Institute of Haematology and Blood Transfusion, Prague.

Isolation and purification of fibrinogen

Fibrinogen was purified from citrated plasma by precipitation with ammonium sulfate using a technique published previously (7). Briefly, fibrinogen was precipitated by the addition of saturated ammonium sulfate to citrated plasma to a final concentration of 25%. Samples were incubated at 4°C for 30 min and then centrifuged at 5,000×g at 4°C for 5 min. Supernatant was removed, the pellet was resuspended in 25% ammonium sulfate solution, and a second ammonium sulfate precipitation was done. Finally, the pellet was solubilised in Tris-HCl buffer, pH 7.4, and the isolated protein was frozen at -80°C.

Coagulation tests

Routine coagulation tests (activated partial thromboplastin time - APTT, prothrombin time - PT, thrombin time - TT, and functional fibrinogen) were performed with citrated plasma samples on a STA-R coagulation analyzer (Diagnostica Stago, Asnieres sur Seine, France). Measurements were made using Diagnostica Stago kits - STA® PTT Automate for the APTT (containing cephalin, thromboplastin, silica, and calcium chloride), STA® Thrombin for the TT (containing thrombin 1.5 NIH U mL⁻¹ and calcium chloride), STA® Fibrinogen for the kinetic fibrinogen assay (containing thrombin 100 NIH U mL⁻¹ and calcium chloride), and STA® Neoplastin CI Plus for the PT (containing thromboplastin and calcium chloride). The immunoturbidimetric assay was performed on a UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan) using anti-human fibrinogen goat antiserum, according to the kit’s manufacturer (K-ASSAY Fibrinogen, Kamiya Biochemical, Seattle, WA, USA).

Fibrinopeptides release and HPLC analysis

The plasma antigenic fibrinogen level was diluted to 1 g L⁻¹ with 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 2 M o-phenanthroline hydrate was added to a final concentration of 0.01 M. For determination of the time dependence of fibrinopeptide cleavage, the plasma fibrinogen samples were incubated with thrombin (0.9 NIH U mL⁻¹ final thrombin activity) at room temperature for 0, 0.5, 1, 3, 5, 10, 20, 30, and 60 min. The reaction was
stopped by the addition of 10% trichloracetic acid and centrifuged at 37,500×g for 30 min at 4°C. Fibrinopeptides in the supernatant were determined by the RP-HPLC performed on a CGC SGX C18 (150 x 3 mm) column (Tessek, Prague, Czech Republic), according to Suttnar et al. (8).

**Thrombin/Reptilase-catalysed fibrin polymerisation**

Fibrinogen polymerisation was measured both in plasma and isolated fibrinogen samples of the Vizovice and control fibrinogen as described earlier (9). Briefly, plasma fibrinogen and isolated fibrinogen levels were diluted to the same antigenic concentration (0.25 g L⁻¹, determined by immunoturbidity) with a 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl. Moreover, control fibrinogen was diluted to the same functional concentration of fibrinogen Vizovice with respect to their different clottability, as determined by the Clauss method. The samples were incubated with thrombin (0.9 NIH U mL⁻¹ final thrombin activity) or reptilase (625x diluted TEClot BAT Reptilase; TECO, Neufahrn, Germany) at 37°C. The turbidity induced by the formation of a clot was monitored at 350 nm in 20 second intervals for 40 min at 37°C using a Synergy HT spectrophotometer (BioTek Instruments, Winooski, VT, USA).

**Fibrinolysis**

Fibrinolysis was measured both in plasma and isolated fibrinogen samples (1 g L⁻¹ final fibrinogen level, determined by immunoturbidity) of the Vizovice and control fibrinogen after the addition of human thrombin (0.5 NIH U mL⁻¹ final concentration), human plasminogen (0.15 U mL⁻¹ final concentration), CaCl₂ (8 mM, final concentration) and human tPA (0.3 µg mL⁻¹ final concentration). Moreover, control fibrinogen was diluted to the same functional concentration of fibrinogen Vizovice with respect to their different clottability, as determined by the Clauss method. The progress of the reaction was followed by measuring the increase and subsequent decrease in turbidity at 350 nm, using a Synergy HT spectrophotometer.

**Isolation of genomic DNA and DNA sequence analysis**

Genomic DNA was isolated from citrate-anticoagulated blood according to Miller et al. (10). All exons of the three fibrinogen genes (FGA, FGB, FGG) were amplified by PCR. Seven sets of primers for the Aα chain exons, eight sets of primers for the Bβ chain exons, and eight sets of primers for the γ chain exons were used. Dideoxy sequencing of all genes was performed using Dye Termi-
Fibrinogen samples were treated with SDS and reducing agent buffer (62.5 mM Tris-HCl buffer, pH 6.8, 4% SDS, 10% mercaptoethanol, 0.05% bromophenol blue, and 20% glycerol). After 5 min incubation at 100°C, samples were loaded onto the gel and separated using SDS-PAGE (7.5% resolving and 3.75% stacking gels, 5°C, 30 mA/gel) under reducing conditions.

The gels were stained with colloidal Coomassie blue stain (Serva, Heidelberg, Germany) for at least 24 hours (h) and destained with 1% acetic acid.

Scanning electron microscopy (SEM)
Fibrin networks were prepared in polystyrene shallow wells. Plasma from either the healthy donor or the patient was mixed with thrombin (2 NIH U mL⁻¹ final concentration) and incubated at room temperature for 3 h. The networks were washed with PBS (Phosphate buffered saline), then with water, and subsequently dehydrated with a series of water-ethanol solutions with increasing ethanol concentration (0, 25, 50, 75, and 100%). Finally, the samples were dried by the CO₂ critical point method (Balzers CPD 010; Balzers, Liechtenstein) and coated with 4 nm thick platinum by sputtering (Balzers SCD 050; Balzers) (11). A TESCAN Vega Plus TS 5135 electron microscope (TESCAN, Brno, Czech Republic) was used for the scanning observations. Images were evaluated using ImageJ data analysis software.

Platelet isolation and aggregation
Washed blood platelets were isolated from the healthy donor by differential centrifugation of blood collected into an ACD, as described earlier (12, 13). Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 250×g at 37°C for 15 min. PRP with the addition of prostaglandin E1 (1 µM final concentration) was incubated in a water bath for 10 min at 37°C and centrifuged at 1,000×g for 10 min at 37°C. The platelet pellet was resuspended in a Ca²⁺ free Tyrode’s buffer in the presence of 1 µM prostaglandin E1 and centrifuged at 600×g for 10 min at 37°C. Finally, the platelet pellet was resuspended in a Tyrode’s buffer to a final concentration of 2 x 10⁸ mL⁻¹, and incubated at 37°C for 30 min. The platelet count in PRP was estimated by an Onyx Coulter Counter blood counter (Beckman Coulter). Platelet aggregation was measured using a PAP-4D aggregometer (Bio/Data Corporation, Horsham, PA, USA). Platelets were mixed with diluted plasma and isolated fibrinogen of either the healthy control or the patient. Fibrinogen concentration was adjusted to 1 g L⁻¹, as determined by

Figure 3: Fibrinolysis curves of fibrin clots. A) The polymerisation and subsequent dissolution of fibrin clots generated from isolated patient’s fibrinogen (red curve) and isolated control fibrinogen (black and green curves) were studied in reaction mixtures containing 0.5 NIH U/ml human thrombin, 0.3 µg/ml human tPA, 8 mM Ca²⁺, 0.15 U/ml human plasminogen, and fibrinogen. Control fibrinogen concentrations were adjusted to the same antigenic and functional levels of fibrinogen Vizovice (black and green curves, respectively). The progress of the reaction was measured by absorbance at 350 nm. The inset shows descending slopes of the fibrinolysis curves normalised for maximal turbidity.
immunoturbidimetry, using Tris-HCl buffer for both the patient and healthy control. Platelet aggregation was initiated with TRAP (thrombin receptor activating peptide; 10 µM final concentration), collagen (0.19 mg mL⁻¹ final concentration), or ADP (2 µM final concentration) and measured at 37°C. All agonists were purchased from Bio/Data Corporation.

Molecular modelling
Molecular modelling was performed using PyMOL Molecular Graphics System software (DeLano Scientific, San Francisco, CA, USA). A crystal structure of human fibrinogen previously published by Kollman et al. (14) (3GHG.PDB) was used in this study.

Results
Patient
The carrier of the Vizovice mutation was a 42-year-old woman from the Czech Republic. Repeated routine coagulation tests (n = 3) showed that activated partial thromboplastin time (aPTT; 33.0 ± 0.6 s), prothrombin time (PT; 13.8 ± 0.5 s), and thrombin time (TT; 15.0 ± 0.4 s) were within normal ranges. At the same time, functional fibrinogen level (1.66 ± 0.11 g L⁻¹) was decreased while immunoturbidimetric fibrinogen level was found to be within the normal range (2.89 ± 0.18 g L⁻¹). Such a discrepancy may be explained by previous reports that the differences in thrombin concentrations and dilutions of plasma in TT and functional fibrinogen test may exert a different effect on various fibrinogen variants (15, 16).

The patient had two consecutive abortions, no bleeding symptoms apart from mild menorrhagia, and no history of thrombosis. As pregnancy loss is more common in women with heritable thrombophilia, microthrombi and infarctions are frequently found in their placentae, the history of two abortions could be associated with a tendency for thrombosis. The patient did not smoke, she had no evidence of diabetes and other common thrombotic risk factors were negative (including Factor V Leiden mutation and prothrombin G20210A). No other hereditary thrombophilia was found in the patient. No other family members were available to the study.

SDS-PAGE and DNA analysis
SDS-PAGE indicated no difference in electrophoretic migration between normal fibrinogen chains and the purified fibrinogen Vizovice chains (data not shown). DNA sequencing revealed a novel natural heterozygous 4161 T>A point mutation in exon 3 of FGA, changing Aα98 Phe to Ile. No other mutations were found. The absence of the identified mutation in the general Czech population was verified by the investigation of 100 control chromosomes of healthy unrelated control subjects.

Fibrinopeptides release and fibrin polymerisation
The kinetics of fibrinopeptides release from fibrinogen Vizovice determined by RP-HPLC was found to be normal (data not shown). Thrombin-induced fibrin polymerisation showed essentially the same rate but substantially decreased final turbidity of fibrinogen Vizovice, when compared with normal fibrinogen, both at the same antigenic and functional levels. Maximum turbidity of both isolated fibrinogen Vizovice and plasma Vizovice was about one-third of that of normal fibrinogen at the same antigenic concentration (Figure 1). Moreover, maximum turbidity of normal fibrinogen adjusted to the functional level of fibrinogen Vizovice reached about 60% of that of normal fibrinogen (Figure 1).
Figure 5: Aggregation responses of platelets in the presence of TRAP (A), ADP (B), and collagen (C). TRAP (10 µM final concentration), ADP (2 µM final concentration), or collagen (0.19 mg/ml final concentration) were added to 2 x 10^8/ml washed platelets in the presence of isolated fibrinogen Vizovice (gray curve) and control fibrinogen (black curve). The plotted curves represent averaged values from three individual measurements with standard deviation.
Fibrinolysis

Lysis of the clots prepared both from Vizovice plasma and isolated fibrinogen Vizovice was found to be impaired. The complete dissolution of Vizovice clots prepared from plasma was achieved after 1,500 s. Normal clots prepared from normal plasma at the same antigenic and functional fibrinogen levels were completely dissolved after 1,000 s and 550 s, respectively (Figure 2). To obtain a better comparison, the lysis curves were normalised for maximal turbidity (inset in Figure 2). The lysis rate of Vizovice clot prepared from the patient’s plasma was almost four times slower than that of a clot prepared from normal plasma (at the same antigenic and functional levels). Similar results were observed with fibrinogen isolated from the patient’s and normal plasma (Figure 3).

Scanning electron microscopy (SEM)

Vizovice fibrin was formed by thinner fibrin fibres and the fibrin network was denser than in a normal (control) fibrin clot, as revealed by SEM (Figure 4). In addition, the Vizovice fibrin clot was formed by shorter curled fibres which were absent in normal fibrin. The average fibrin fibre thickness for fibrin Vizovice was found to be 82 ± 26 nm (mean ± SD), while the average thickness of normal fibrin fibres was 211 ± 38 nm. The fibrin network significantly differed from that of the control at the level of significance p ≤ 0.05 (determined from three independent clots, n = 100).

Platelet aggregation

The interaction of platelets with fibrinogen Vizovice was investigated using platelet aggregometry. Aggregation was initiated using three different agonists (TRAP, ADP, and collagen) and significant differences were found. The aggregation of platelets was increased both in the presence of patient plasma and isolated fibrinogen Vizovice, when compared to the control normal plasma and fibrinogen. Aggregations curves for all three agonists in the presence of isolated fibrinogen Vizovice are shown in Figure 5. Plotted curves represent averaged values from three individual measurements with standard deviation (SD).

Discussion

We identified a novel natural heterozygous mutation Phe98 to Ile in the Aα chain of fibrinogen and designated it as fibrinogen Vizovice. The mutation was associated with an impaired fibrin poly-
merisation and fibrinolysis and enhanced platelet aggregation after activation by TRAP.

The amino acid substitution is located in the middle of the coiled-coil connector of fibrinogen in the RGDF sequence Aa95-98. The mutation is situated in close proximity to the plasmin cleavage site and the cryptic site, which affects the lateral association of protofibrils (17). The coiled-coil connector of fibrinogen is a conserved structure among all vertebrates, implying that a single amino acid substitution in its structure may affect fibrinogen function. The coiled-coil connector is not only a passive structure but plays a significant role in fibrin formation and morphology. It has been recently described that several amino acid residues of the coiled-coil connector are involved in protofibril lateral association, most of them located in the Bβ chain: Bβ111 (4), Bβ118-134 (18), Bβ158-160 (19), Bβ166 (20) and Aa52-78 (21), and y114 (22).

Aa98Phe is situated in the helix (▶ Figure 6) formed by residues 97Asp-110Arg and is directed inwards in the coiled-coil region (7, 23). Its replacement by isoleucine residue may lead to an insufficient amount of stabilising interactions and uncompensated entropic cost associated with the folding of the coiled-coil connector. This may cause conformational changes affecting both the structure and function of the region. Replacement of the bulky aromatic side chain of phenylalanine by an aliphatic side chain of isoleucine may also disrupt π–π stacking interaction with aromatic amino acids residues in its neighbourhood (Trp at position ββ125 and Tyr at position y68) (24). Molecular modelling revealed decreased interaction of Aa 98Ile with its neighbours compared to interaction of Aa 98Phe. This may affect lateral aggregation of protofibrils and may lead to curly short fibrin fibres, observed by SEM (▶ Figure 4). Similar clot morphology was obtained by Marchi et al. in fibrinogen variant Lima with extra glycosylation in the coiled-coil connector (25).

The Aα chain of fibrinogen contains two RGDX sequences known to mediate platelet and endothelial cell interaction with fibrinogen, one at a position of Aa95-98 and another one at Aa572-575. It is known that the RGD sequence mediates interaction of fibrinogen with platelet receptor GPIIb-IIIa and αβ, receptor expressed on different cells. The first RGDX sequence is present in the coiled-coil region adjacent to the observed point mutation. The second sequence plays an important role in wound healing, angiogenesis, and platelet aggregation. However, some studies have shown that the Aa95-97 RGD motif does not play a significant role in initial platelet and endothelial cell adhesion (26).

Several previous studies have shown that the RGD sequence can interact with different receptor sequences on fibrinogen (20). The substitution Aa98Ile seems to be a direct cause of thrombotic complications in the patient. Similar observations have been reported by Kotlin et al. with fibrinogen Plzeň, and Morris et al. with fibrinogen San Diego IV where a point mutation in Aa106 Asn to Asp and 69Leu to His in the coiled-coil connector decreased the fibrinolytic rate and final turbidity (22, 28). Moreover, abnormal fibrin clot structure and delayed lysis are often connected with thromboembolism (29).

In conclusion, we report here a novel natural heterozygous fibrinogen variant characterised by a point mutation, AaPhe98Ile, in the coiled-coil connector. The substitution plays an important role in the lateral aggregation of protofibrils, fibrinolysis, and platelet aggregation, and may be the direct cause of the thrombophilia leading to abortions in the patient. Experiments with naturally occurring dysfibrinogens from patients provide clues to the struc-
ture-function relationships and significant correlations with clinical consequences.

Conflicts of interest
None declared.

References