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Normal Plasmic Cleavage of the γ-Chain Variant of "Fibrinogen Saga" with an Arg-275 to His Substitution

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Key words

Fibrinogen Saga – γ-Arg → His substitution – Dysfunctional fragment D_1 – Calcium protection from plasmin

Summary

We have identified a γ-Arg-275 to His substitution in an abnormal fibrinogen designated as "fibrinogen Saga" characterized by impaired fibrin monomer polymerization. By chromatofocusing chromatography, we isolated normal and abnormal fragment D_1 populations separately from the plasmic-calcium digests of fibrinogen derived from the propositus, a heterozygote for the abnormality. We found that both normal and abnormal fragment D_1's were similarly protected from digestion by plasmin in the presence of calcium ions and further degraded to fragments D_2 and D_3 due to cleavage of the γ-chain remnant when calcium ions were replaced by chelating agents. Abnormal fragment D_1 failed to inhibit both thrombin-clotting of normal fibrinogen and polymerization of normal fibrin monomer, while normal D_1 exhibited marked inhibitory activities. In an aberrant peptide comprising residues γ-274–302 isolated by HPLC from the lysyl endopeptidase-digests of abnormal fragment D_1, we identified a His substituting for an Arg at position 2, which corresponds to position 275 of the mutant γ-chain.

Introduction

It has been recently reported that the γ-chain remnant of fragment D_1 (γ/D_1) derived from an abnormal fibrinogen designated as fibrinogen Haifa was not protected by calcium against plasmin (1). Although direct evidence has not been provided, an amino acid substitution of an Arg by a His is suggested at position 275 of the γ-chain derived from the abnormal molecule. This mutation site resides indeed near the plasmic cleavage site, i.e., the carboxyl side of γ-Lys-302, which should be protected from plasmin when calcium binds to the putative binding site allocated to the carboxy terminal region of the γ-chain. The mutation may thus render the cleavage site of the mutant γ-chain susceptible to plasmin by altering the calcium-dependent protection from plasmin.

Recently we have identified the same type of amino acid substitution in an abnormal fibrinogen found in a Japanese family. In this fibrinogen, however, we observed normal protection of the mutant γ/D_1 by calcium against plasmin, although abnormal fragment D_1 separated from normal D_1 was obviously devoid of the polymerization site(s) assigned to the D domain of fibrinogen.

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Materials and Methods

Collection of Blood

Blood was collected by clean venipuncture and immediately anticoagulated with 1/9 vol of 3.8% trisodium citrate. Plasma was separated by centrifugation at 3,000 × g for 30 min at 22° C.

Purification of Fibrinogen

Fibrinogen was purified from plasma by repeated 25% ammonium sulfate saturation after passing through gelatin-Sepharose and lysine-Sepharose columns connected in tandem as reported previously (2).

The purity of fibrinogen was more than 94% as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of Laemmli (3).

Studies on Fibrinogen and Fibrin Monomer

The thrombin and Ancrod times and clottability tests were performed essentially as described (2). Gross structure of subunit polypeptides and cross-linking mediated by activated factor XIII (XIIIa) were examined by SDS-PAGE of Laemmli after reduction using 10% gels.

Fibrinopeptides A and B released upon treatment with thrombin or Ancrod were analyzed by reverse-phase HPLC (4).

Aggregation of fibrin monomer was studied essentially according to Gralnick et al. (5).

Digestion of Fibrinogen by Plasmin in the Presence of Calcium Ions and Isolation of Abnormal Fragment D_1 from the Digests

Twenty mg of fibrinogen was digested with plasmin (a mixture of 104 μg of plasminogen and 3,200 units of streptokinase, Kabi Vitrum, Stockholm, Sweden) in the presence of 2 mM $CaCl_2$ essentially as described by Southan et al. (6). The plasmic digests were dialyzed against 0.025 M Tris-HCl, pH 7.8 and subjected to chromatofocusing essentially as described by Masci et al. (7). The flow rate was maintained at 20 ml/h and 4.5-ml fractions were collected. Fractions enriched with fragment D_1 which had been identified by elution profiles and SDS-PAGE were collected and combined as indicated by horizontal bars I and II (see Results and Fig. 2). They were concentrated and dialyzed against 0.05 M Tris-HCl, pH 7.4 containing 0.1 M NaCl (TBS) by ultrafiltration using a membrane, CX-30 (Millipore Co., Bedford, MA, USA) and examined for inhibition of thrombin-clotting of normal fibrinogen and polymerization of normal fibrin monomer.

Digestion of Fragment D_1 by Plasmin in the Presence of Calcium Ions or Ethylene Glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic Acid (EGTA)

Portions of these peak fractions were also tested for susceptibility to plasmic digestion. Namely, 40 μg each of peak I and peak II fractions (1 mg/ml) in TBS containing either 5 mM $CaCl_2$ or 10 mM EGTA was further digested with plasmin at 37° C by additions of 0.416 μg of plasminogen and 12.8 units of streptokinase, and products were analyzed by SDS-PAGE of Laemmli using 10% gels.

Lysyl Endopeptidase Digestion of Fragment D_1

Both normal and abnormal fragment D_1's were separately dissolved at 0.5% in 0.5 M Tris-HCl, pH 8.5 containing 6 M guanidine-HCl and

10 mM ethylenediaminetetraacetate (EDTA). They were reduced, S-pyridylethylated as described elsewhere (8) and dialyzed against distilled water. The S-alkylated proteins, precipitated during dialysis as the detergent was removed, were collected by centrifugation and dissolved in 3 M urea in 0.05 M Tris-HCl, pH 9.0 at a concentration of 0.2% (w/v). They were digested with lysyl endopeptidase (Wako Chemical Co., Osaka, Japan) (enzyme/substrate ratio = 1/25, w/w) for 8 h at 37° C and analyzed by reverse-phase HPLC on a Cosmosil 5C18P column by applying a linear gradient of 0% to 50% of acetonitrile in 0.1% trifluoroacetic acid. The column effluent was monitored by an absorbance at 210 nm.

Total Amino Acid and Sequence Studies

Amino acid sequence was analyzed by a gas-phase sequencer, Applied Biosystems 470A Protein Sequencer connected to 120A PTH Analyzer (Applied Biosystem, Foster City, CA). Total amino acid analysis was performed by the phenylthiocarbamyl method (9) on the PICO TAG system (Waters, Millipore Corp., Milford, MA).

Results

Patient and her Family

A 16-year-old female with hematuria was studied for routine coagulation and found to have markedly prolonged thrombin time (23.4 sec, control 9.4 sec) and Ancrod time (82.4 sec, control 11.3 sec), and apparently reduced plasma fibrinogen (<40 mg/dl, normal 150–350 mg/dl) when determined by the thrombin time method of Clauss (10). When plasma fibrinogen was determined by other methods, normal values were obtained (345 mg/dl by the turbidity method (2) and 245 mg/dl by a modification of the method of Ratnoff and Menzie (11), in which fibrinogen was precipitated by heat-treatment at 56° C for 5 min instead of clotting with thrombin). The prothrombin time was slightly prolonged (13.9 sec; control 11.4 sec). The marked prolongation of thrombin and Ancrod times were, however, considerably shortened by additions of $CaCl_2$ (thrombin time 9.4 sec, control 6.9 sec and Ancrod time 13.9 sec, control 12.8 sec).

Similar data were obtained on plasma derived from her father but not on plasmas derived from her mother and a brother (data not shown).

These data indicate that the patient is most likely to be a heterozygote for an abnormal fibrinogen inherited from her father.

Studies on Purified Fibrinogen

The gross structure and cross-linking profiles mediated by XIIIa were essentially identical with those of normal fibrinogen when examined by SDS-PAGE (profiles not shown).

The thrombin and Ancrod times were, however, markedly prolonged (thrombin time 114 sec, control 14.7 sec and Ancrod time >180 sec, control 11.3 sec). They were considerably shortened by additions of $CaCl_2$ (thrombin time 46.1 sec, control 7.9 sec and Ancrod time 37.2 sec, control 8.4 sec).

Releases of fibrinopeptides A and B were normal as evidenced by SDS-PAGE and HPLC (profiles not shown). Aggregation of fibrin monomer derived from the propositus was markedly altered (Fig. 1).

Digestion of Fibrinogen with Plasmin in the Presence of Calcium Ions and Separation of Fragment D_1 by Chromatofocusing

Purified fibrinogen was digested with plasmin in the presence of 2 mM $CaCl_2$ and subjected to chromatofocusing chromatography. As depicted in Fig. 2, fragment D_1 was eluted in a sharp peak

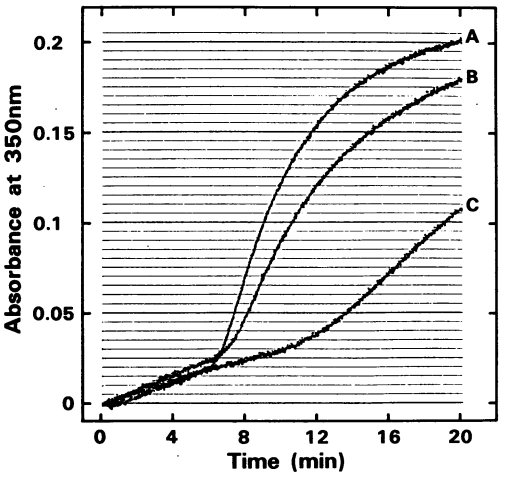

Fig. 1 Polymerization of fibrin monomer. N: normal fibrin monomer; P: patient's fibrin monomer. Polymerization was continuously monitored by an absorbance at 350 nm (A_{350}) after 26-fold dilution of fibrin monomer (A_{280} = 1.6) with 0.06 M phosphate buffer, pH 6.8

with a rather broad shoulder on its descending limb at pH between 5.8 and 4.7. The peak fractions eluted at a position identical with normal fragment D_1 and the shoulder fractions were separately collected as indicated by horizontal bars (peaks I and II, respectively), and studied for inhibition of thrombin-clotting of normal fibrinogen and polymerization of normal fibrin monomer.

As summarized in Table 1, thrombin-clotting of normal fibrinogen was prolonged dose-dependently by additions of peak I whereas it was only slightly prolonged by peak II. Peak I also inhibited polymerization of normal fibrin monomer while peak II did not do so (Fig. 3). These data indicate that the polymerization site assigned to the D domain of fibrinogen functions normally in peak I but not so in peak II. We thus presumed that peak I was derived from the normal fibrinogen population and peak II from the abnormal one in this heterozygous fibrinogen.

Further Digestion of Abnormal D_1 in the Presence of Calcium Ions or EGTA

We digested the isolated abnormal fragment D_1 with plasmin in the presence of 5 mM $CaCl_2$ or 10 mM EGTA and analyzed the digests by SDS-PAGE after reduction. The γ-remnant of abnormal D_1 was substantially protected from plasmin in the presence of calcium (γ/D_1, lanes 1–3 in panel B, Fig. 4) and sequentially degraded in a normal fashion to those of fragments D_2 and then D_3 when $CaCl_2$ was replaced by 10 mM EGTA (γ/D_2 and γ/D_3, lanes 4–8 in panel B, Fig. 4).

Detection of an Aberrant Peptide in the Lysyl Endopeptidase-Digests of Abnormal Fragment D_1

Fig. 5 depicts HPLC elution profiles of the lysyl endopeptidase-digests of reduced and S-pyridylethylated normal and abnormal fragment D_1's (panels A and B, respectively). As indicated by an arrow in panel B, there was an aberrant peptide K 26′, eluted slightly earlier than the normal counterpart, K 26, shown in panels A and B. The K 26′ and K 26 peptides were collected separately and analyzed for the primary sequence of the first five cycles and for amino acid compositions.

As shown in Table 2, a His residue was identified at position 2 in peptide K 26′, for an Arg residue in peptide K 26. On the basis of the known sequence data on the subunit polypeptides of human fibrinogen (12–15), these peptides were assigned to the γ-274–302

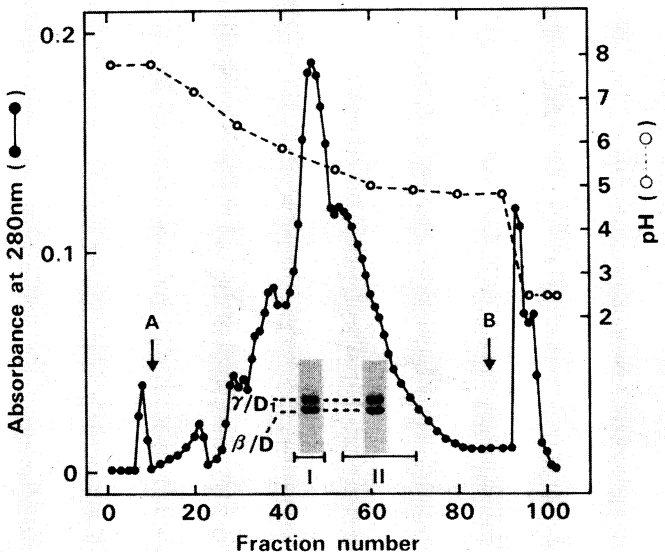

Fig. 2 Chromatofocusing chromatography of the plasmic digests of patient-derived fibrinogen prepared in the presence of 2 mM $CaCl_2$. Proteins were eluted first with 360 ml of Polybuffer 74, pH 4.9 (arrow A) and then with 80 ml of 0.025 M glycine-HCl, pH 2.5 (arrow B). Fragment D_1-enriched fractions confirmed by elution profiles and SDS-PAGE were separately collected as indicated by horizontal bars I and II, respectively. Inset. SDS-PAGE of the combined fractions, peaks I and II, after reduction. 10% gels

Table 1 Inhibition of thrombin-clotting of normal fibrinogen by peak I and peak II fractions separated by chromatofocusing chromatography of plasmic digests of the propositus' fibrinogen

Molar ratio (D_1/Fbg*)	Thrombin time (s) Peak I	Peak II
0	16.6	16.6
1	21.8	16.5
2	31.0	17.1
3	37.8	20.1
4	64.3	20.4

* Fbg: fibrinogen.

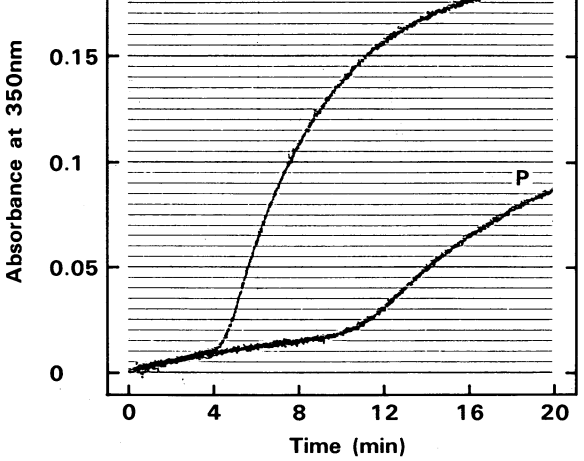

Fig. 3 Inhibition of normal fibrin polymerization by isolated fragment D_1-enriched fractions, peaks I and II. A: normal fibrin monomer alone (NF); B: addition of the peak II fraction to NF; C: addition of the peak I fraction to NF. Twenty μg of normal fibrin monomer in 0.02 M acetic acid (4 mg/ml) was diluted with 500 μl of 0.06 potassium phosphate, pH 6.8 containing either the peak I or peak II fraction at a molar ratio to fibrin monomer = 1:1

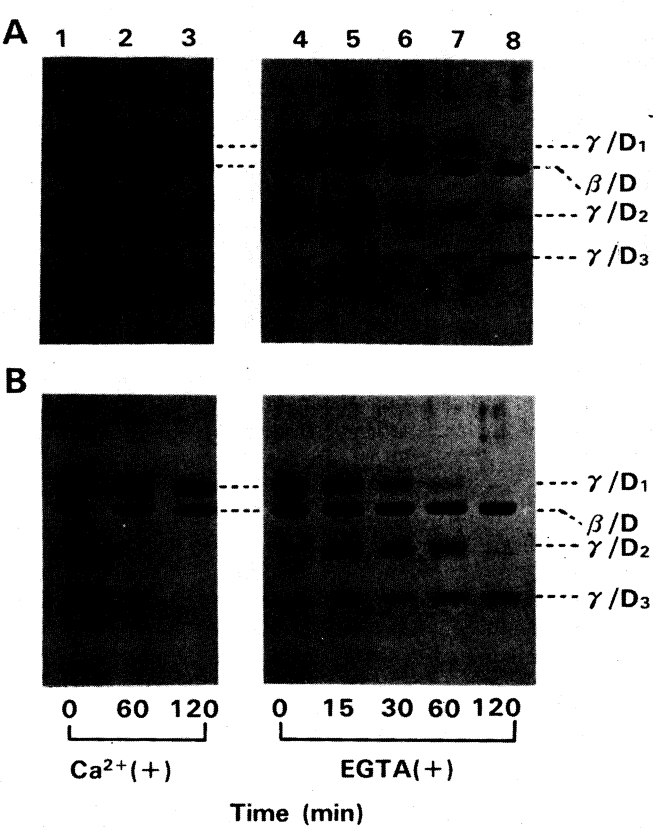

Fig. 4 Further digestion of fragment D_1 with plasmin in the presence of $CaCl_2$ or EGTA. At various intervals as shown, aliquots were removed, treated with 1% SDS and 1% DTT, and analyzed by SDS-PAGE of Laemmli using 10% gels. Note protection from plasmin of the γ-chain remnant of abnormal fragment D_1 (γ/D_1) in the presence of 5 mM $CaCl_2$ (panel B, lanes 1–3), as compared with that of normal (panel A, lanes 1–3). Degradation of γ/D_1 to γ/D_2 and then γ/D_3 in the presence of EGTA were essentially identical with each other (lanes 4–8 in panel A for normal and lanes 4–8 in panel B for abnormal fragment D_1)

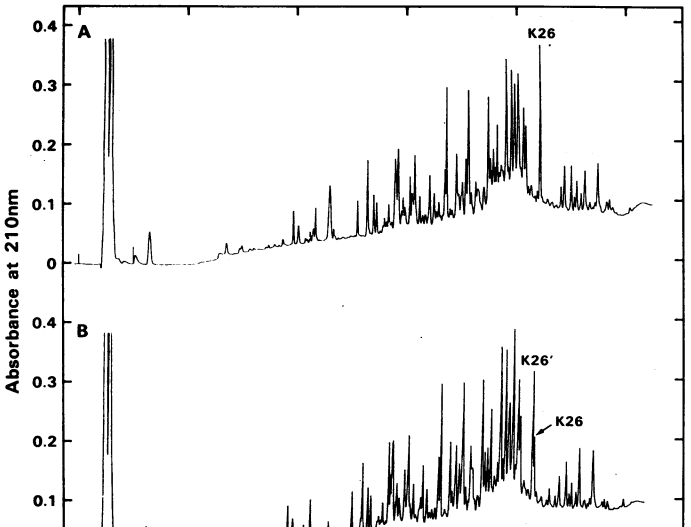

Fig. 5 HPLC elution profiles of the lysyl endopeptidase-digests of reduced and S-pyridylethylated fragment D_1. Reduced and S-pyridylethylated normal (A) and abnormal (B) fragment D_1's were digested with lysyl endopeptidase, and the digests analyzed by HPLC on a Cosmosil 5C18P column. For details see text

Table 2 Amino acid sequence for the first 5 cycles of aberrant peptide K 26′

Cycle	Peptide K 26′ Amino acid	p_{mol}	Normal sequence*
1	Tyr	114	Tyr
2	His	24	Arg
3	Leu	36	Leu
4	Thr	16	Thr
5	Tyr	40	Tyr

* Referred from Chung et al. (14), and Lottspeich and Henschen (15).

Table 3 Amino acid composition of peptides K 26 and K 26′

Amino acid	Peptide K 26 Residues per molecule	Peptide K 26′	Theoretical value*
Asx	4.89	5.09	7
Ser	1.25	1.05	1
Gly	5.40	4.93	5
His	0.09	0.97	0
Arg	1.36	0.19	1
Thr	1.16	0.93	1
Ala	3.77	3.53	4
Pro	1.10	1.17	1
Tyr	3.08	2.85	3
Leu	1.00	0.90	1
Phe	3.91	3.57	4
Lys	1.07	0.96	1

* Referred from Chung et al. (14), and Lottspeich and Henschen (15).

peptide segments. Amino acid compositions of peptides K 26 and K 26′ were examined and compared with theoretical values for the γ-274–302 peptide segment of normal fibrinogen as summarized in Table 3. In support of the sequence analysis, an Arg residue was missing but an His residue was present in peptide K 26′. Amino acid compositions of K 26 were essentially identical with the theoretical values deduced from the known sequence data.

Discussion

An amino acid substitution of a γ-Arg-275 by a His has recently been reported in fibrinogens Bergamo II, Essen and Perugia (16). The same type of amino acid substitution was also suggested in an abnormal fibrinogen, fibrinogen Haifa, characterized by defective protection from plasmic degradation by calcium ions of the γ-chain remnant of fragment D_1 (1). This mutation site is indeed located near the putative calcium-binding region of the γ-chain of fibrinogen comprising γ-303–356 (17) or γ-311–336 (18) amino acid residues and thus an Arg to His substitution may well affect the calcium-dependent conformer of the D domain of fibrinogen.

In an abnormal fibrinogen with impaired polymerization of fibrin monomer found in a Japanese family, we have identified the same type of amino acid substitution in the γ-chain of fibrinogen derived from the heterozygous propositus. Since profiles of the release of fibrinopeptides A and B were both normal, the abnormality was searched for in the D domain of abnormal fibrinogen. We thus digested fibrinogen derived from the propositus with plasmin in the presence of calcium ions and subjected the digests to chromatofocusing chromatography to separate an abnormal fragment D_1 population from a normal one. We could separate the abnormal D_1 population with a dysfunctional polymerization site(s) as confirmed by defective inhibition of thrombin-clotting of fibrinogen (Table 1) and fibrin monomer polymerization (Fig. 3). Studies on the isolated D_1 populations revealed that the γ-chain remnant of abnormal D_1 was protected from plasmic cleavage in the presence of calcium ions, but was further cleaved by plasmin to yield γ/D_2 and then γ/D_3 sequentially when calcium ions were replaced by EGTA. The degradation of the γ-remnants of abnormal fragments D_1 and D_2 may have proceeded slightly faster than those of the normal ones when the corresponding gels were closely compared with each other (lanes 5 and 8 in panels B and A, Fig. 4, respectively).

From the lysyl endopeptidase-digests of abnormal fragment D_1, we isolated an aberrant peptide that corresponds to a peptide segment comprising γ-274–302 amino acid residues and identified an Arg to His substitution at cycle 2, i.e., at position 275 of the mutant γ-chain. No other aberrant peptides or amino acid substitutions were identified in the digests of isolated abnormal fragment D_1.

Recently, we also identified another type of amino acid substitution, i.e., an Arg by a Cys at position 275 of the γ-chain in four abnormal fibrinogens, designated as fibrinogens Tochigi (19, 20), Osaka II (21, 22), Morioka (21) and Tokyo II (2). Fragment D_1's derived from these abnormal fibrinogens were all protected in a normal fashion from further digestion by plasmin when calcium ions were present. Thus it seems to be likely that a point mutation of an Arg to a His or a Cys at position 275 of the γ-chain would not necessarily make the γ-302–303 peptide bond susceptible to plasmin in the presence of calcium ions as shown in fibrinogen Haifa (1), although the mutation at position 275 of the γ-chain may critically affect polymerization site(s) assigned to the D domain of fibrinogen.

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