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INFLUENCE OF FIBRIN D AND DD FRAGMENTS ON FIBRINOGEN AND FIBRINOGEN FRAGMENT X POLYMERIZATION INITIATED BY THROMBIN OR ANCISTRON

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Aim. Study of the role of the complex between the αC region and the BβN domain in the initial stages of fibrin polymerization has been investigated.

Materials and Methods. Method of turbidimetry to study the influence of fibrinogen fragments D and DD on the polymerization and methods of isolation, purification, fragmentation for fibrinogen, monomer and cross-linked fibrin, fibrinogen X-fragment, Glu -plasminogen were used.

Results. It was shown that fragment DD completely inhibited polymerization process in all the systems examined ("Fg + Thr", "Fg + Anc H", "X + Thr", "X + Anc H"). Fragment D inhibited fibrin polymerization at all stages in the system "Fg + Thr", but in the system "Fg + Anc H" it almost did not influence fibrin polymerization. In the both systems "X + Thr" and "X + Anc H" fragment D weakly inhibited the self-assembly of fibrin molecules into protofibrils, but accelerated the process of lateral association in the second system.

Conclusions. The data obtained indicated that the complex between the αC region and the BβN domain of fibrin desA, on the initial stage of polymerization supported the rate of self-assembling and lateral association of fibrin desA protofibrils, protecting the oligomers against the depolymerizing influence of fibrinogen.

Key words: fibrinogen, fibrin, fragment D, fragment DD, fragment X, BβN-domain-αC-region complex.

Fibrinogen is a multidomain protein that is involved in a series of biological processes in an organism: blood clotting and fibrinolysis, wound healing, angiogenesis, inflammation and metastasis.

The polymerization of fibrin is a two-stage process. On the first, enzymatic, stage, thrombin transforms fibrinogen into fibrin desA by cleaving fibrinopeptides A (FpA) from the N-ends of Aα-chains. The removal of fibrinopeptides A exposes the "A"-sites of polymerization ("knobs") complementary to polymerization sites "a" ("holes") located in the γ-module of the D region. Both polymerization sites ("A" and "a") are located on the "facial" surface of fibrin desA molecule [1]. It is considered that interaction between polymerization sites "A" and "a" is a "driving power" for fibrin polymerization — the self-assembling of fibrin molecules into the fibril network of the clot [2]. In the protofibrils having formed out of fibrin desA, the removal of fibrinopeptides B (FpB) by thrombin accelerates significantly and polymerization cites "B" ("B"-"knobs") on the N-ends of β-chains are exposed. The appearing of the sites "B" causes the acceleration of protofibril lateral association due to interaction between the sites "B"-"b" [3].

After fibrinopeptide A removal of fibrinogen molecule and forming of contacts “A”-“a” and “C”-“c” in fibrin desA oligomers, the connection between fibrinopeptide A and the αC-region and the connection between αC regions in fibrin desA molecule are broken down [4, 5]. According to X-ray structural analysis, the region 1–53 of the fibrinogen BβN domain is quite mobile [6]. However, after fibrinopeptide A removal and the DDE triad formation the two sites of the region — fibrinopeptide B (Bβ1–14) and Bβ28–36 — remain connected to the αC region [5, 7. 8]. As result, the domain BβN and the αC domain form a new temporary structure with limited mobility. It includes the region 37–54 of the fibrin Bβ chain, which has the length of about 50 Å and binds this temporary structure to the E module of the molecule. The structure can be supposed to be located between neighboring DDE triads in the protofibril. This assumption was confirmed by the data of the X-ray structural analysis of chicken fibrinogen [9].

An assumption was made that an important role in providing the high rate of protofibril formation is also played by BβN-domain-αC-region complexes, which remain on the surface of the molecule of fibrin desA [7, 8, 10]. The presence of the BβN-domain-αC-region complex at the very beginning of polymerization process, interactions “A”-“a” and D-D-long, the formation of the DDE triad altogether suggest an important role of the complex in fibrin polymerization process. These assumptions were confirmed by the comparative analysis of the polymerization processes of fibrinogen and, lacking for αC regions, fibrinogen fragment X both stimulated by thrombin and ancistron, and by the inhibition of these processes by fibrin fragments D and DD.

Materials and Methods

Fibrinogen was isolated from citrate human blood plasma in the presence of soybean trypsin inhibitor by salting out with Na_2SO_4 [11]. Glu-plasminogen was isolated from donor blood plasma by affinity chromatography using Lys-sepharose [12]. Ancistron — reptilase-like enzyme was isolated from venom of Agkistrodon halys halys (13). Fragment D was purified from the plasmin digest of noncross-linked fibrin by the method of ion-exchange chromatography on CM-Sephadex G-50 (“Pharmacia”, Sweden) in accordance with [14]. D dimer was obtained from the plasmin digest of cross-linked fibrin by the aid of affine chromatography on fibrin-sepharose according to [15]. D dimer was dialyzed against 0.05 M ammonium acetate buffer pH 8.5 and freeze-dried.

Preparation of fibrinogen fragment X. Fragment X was isolated as described in [16] with some modifications. Fibrinogen dissolved in 0.02 M HEPES buffer pH 7.4, 0.15 M NaCl, 1.0 mM $CaCl_2$ was treated with plasmin in molar ratio of 1 mole of plasmin to 1000 mole of fibrinogen. Plasmin was formed immediately in the reaction medium from plasminogen activated by streptokinase in molar ratio of plasminogen to streptokinase as 10:1. The reaction was carried out during 20 min at 25 °C and was stopped by aprotinin in 20-fold molar surplus. The digest was passed through the Lys-Sepharose column, concentrated and applied on the Sephacryl S-300 column 3×100 cm, equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 2 M NaCl, 100 mM 6-aminocaproic acid, 1 mM $CaCl_2$. The fractions containing fragment X_1 according to PAAGE were dialyzed against 0.02 M HEPES buffer pH 7.4 with 0.15 M NaCl, 0.005% NaN_3. The purity of the preparation was tested by ELISA with anti-αC and anti-BβN-domain monoclonal antibodies. The fragment X preparation was frozen and stored at –20 °C.

The effect of D and DD-dimer fragments on the fibrinogen polymerization, initiated by thrombin or ancistron, was studied with turbidimetry method using spectrophotometer SF 2000 at 350 nm (17, 18).

Results and Discussion

To clarify the role of the BβN-domain- C-region complex in fibrin polymerization process, we compared the rates of the polymerization of fibrin desA and desAB forms, which that either contained this complex or did not and the influence of polymerization inhibitors (fragments D and DD) on the separate stages of the polymerization process.

The BβN-domain-αC-region complex was present in the native fibrinogen molecule but was absent in fibrinogen fragment X, causing higher maximal clot turbidity level for the polymerization of fibrinogen in both thrombin (Thr) and ancistron H (Anc H) containing systems (Fig. 1.1, 1.2). In the system “Fg + Thr”, fragment D inhibited significantly all the stages of fibrin polymerization: protofibrill self-assembling, lateral association of protofibrils and the reaching of the maximal

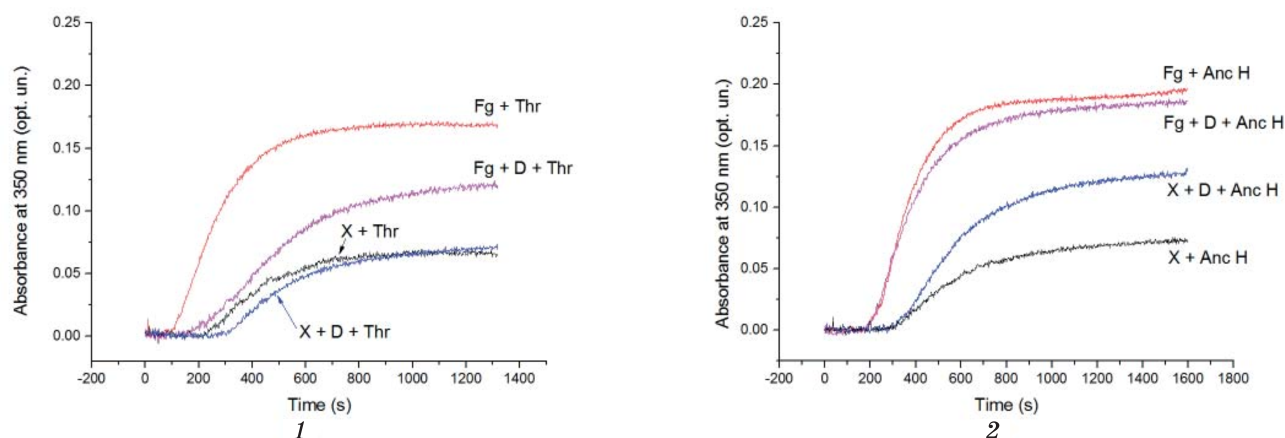

Fig. 1. **Influence of fragment D on polymerization of fibrinogen and fibrinogen fragment X initiated by thrombin (*1*) and ancistron (*2*) in reaction medium containing 0.02 M HEPES, pH7.4, 0.15 M NaCl and 1 mM $CaCl_2$**

Concentration of fibrinogen was 0.3 μM, fibrinogen fragment X — 0.3 μM, fibrin fragment D — 1.8 μM, thrombin and ancistron — 0.1 NIH/ml.

clot turbidity level (Fig. 1.1). Thrombin, having removed fibrinopeptide A, started to remove fibrinopeptide B after a short delay connected with the formation of protofibrils, which resulted in the degradation of the complex formed by the BβN domain and the αC region and thus the increasing of the accessibility of polymerization sites "A", "B", "C" for fragment D. In the system "Fg + Anc H", fragment D almost did not make an influence on fibrin polymerization process, because the complex of the BβN domain and the αC region remained in this system and protected polymerization sites A and C against inhibition by fragment D. Additionally, in the complex mentioned, the αC regions promoted the self-assembling of fibrin molecules into protofibrills and the lateral association of the last ones. In the system "X + Thr" (Fig. 1.1), fragment D weakly inhibited the self-assembling of fibrin molecules into protofibrils and did not influence the process of lateral association. In the fragment desA-X, the D region interacted with the E region more effectively than isolated fragment D, because this interaction carried out by two pairs of polymerization sites, "A"-"a" and "C"-"c", while the interaction with fragment D was due to one pair of polymerization sites. In the system "X + Anc H" (Fig. 1.2), while fibrinopeptide B in fragment X remained intact, fragment D weakly inhibited the self-assembly of fibrin molecules into protofibrils but accelerated the lateral association of protofibrils. This could be explained by the inclusion of fragment D into the structure of a clot, which was mediated by the presence of fibrinopeptide B and/or primary lateral association γC-γC contacts of D-fragments (1). The absence of αC region in fragment X and the desintegration of the complex of the BβN domain and the αC region in fibrin desA resulted in the deceleration of protofibril formation and lateral association, which indicated an important role of the complexes in these processes.

Fragment DD completely inhibited polymerization process in all the systems examined ("Fg + Thr", "Fg + Anc H", "X + Thr", "X + Anc H"), evidently, due to the presence of three pairs of polymerization sites (2a, 2b i 2c) localized in a single molecule and thus providing highly affine interaction between fragment DD and fibrin desA or fragment X on the very initial stages of polymerization. It is also essential that the binding of the DD fragment to the E region of the desA or desAB fibrin molecules blocks the end-to-end interaction of the D regions of the fibrin molecules at formation the basic structures of the protofibril — the DED triads.

In contrast to fragment D, which has three polymerization sites (a, b, c), fragment DD has a double number of these sites (2a, 2b, 2c) and thus completely blocks polymerization process of either fibrin desA or fibrin fragment X by competing effectively with the D regions of fibrin or fragment X for polymerization centres A, B and C.

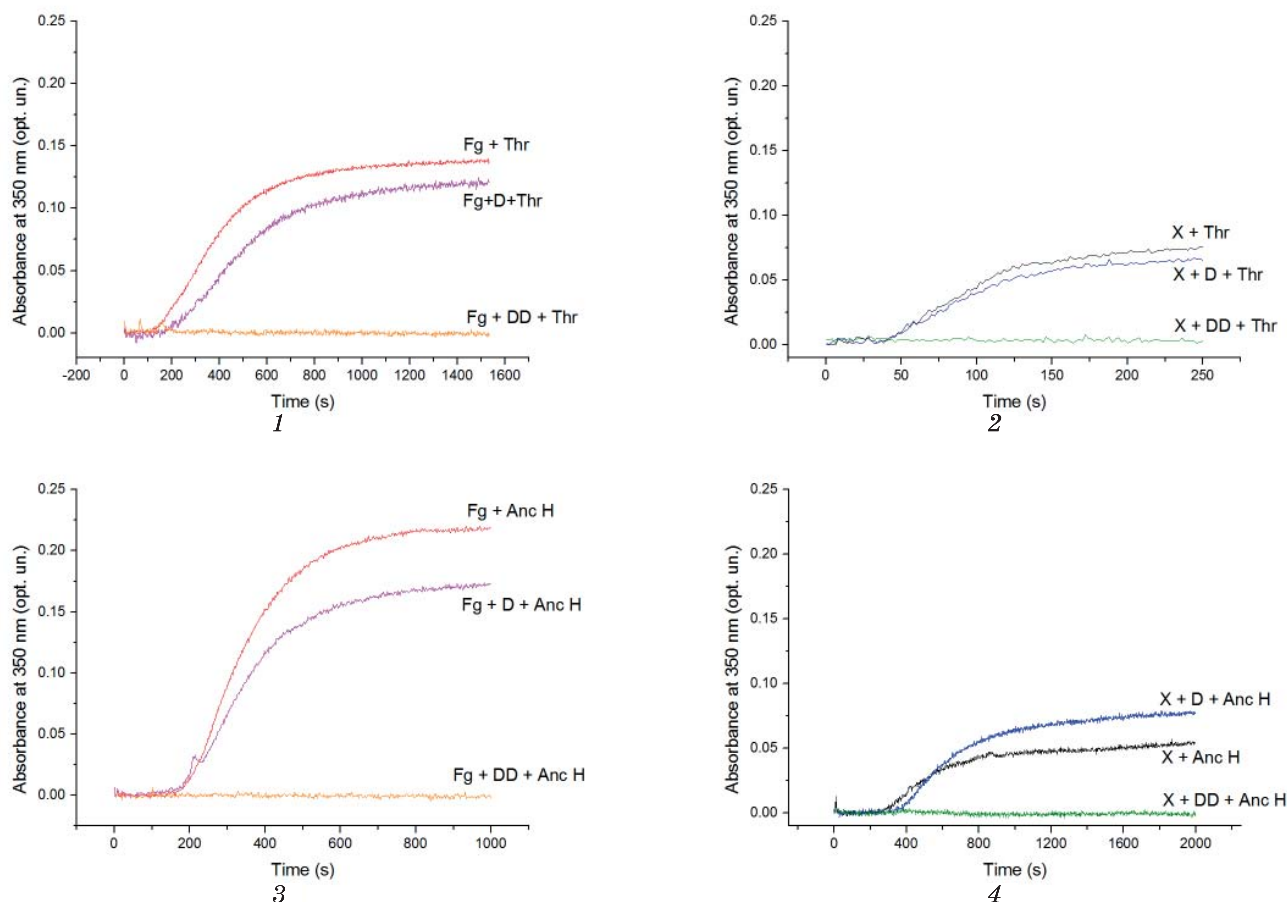

Fig. 2. **Comparison of the effects of fragments D and DD on initiated by thrombin and ancistron of fibrinogen polymerization**

Reaction medium contained 0.3 μM fibrinogen and fibrinogen fragment X — 0.3 μM, fibrin fragment D — 1.8 μM of each, in 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$. Concentration DD — 0.9 μM, thrombin and ancistron — 0.1 NIH/ml.

Conclusions

In fibrin desA, when, previously connected to the αC regions, fibrinopeptides A are removed, the complex between the BβN domain and the αC region becomes mobile and able to give access to polymerization sites on the molecule for other fibrin molecules or oligomers for further interactions. The mobility of the complex may be confirmed by the fact that fragment D inhibits polymerization of fibrin only partially and fragment DD does it completely in all the cases examined, which indicates the displacement of the complex by a more affine inhibitor. According to the evidence obtained, present only in fibrin desA molecules, the complex favours the interaction exactly of fibrin desA molecules with each other resulting in the formation of the DDE triad and promotes further protofibril formation and the lateral association of the protofibrils. Thus, the complex plays an important role in the growth of fibrin protofibrils as the result of the process of fibrin desA polymerization.

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Conflict of interest

Authors declare no conflict of interest.

REFERENCES

1. *Yang Z., Mochalkin I., Doolittle R. F.* A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. *Proc. Natl. Acad. Sci. USA*. 2000. 97(26), 14156–14161.
2. *Weisel J. W., Litvinov R. I.* Fibrin Formation, Structure and Properties. *Subcell Biochem*. 2017, 82, 405–456.
3. *Wiltzius P., Dietler G., Kanzig W., Haberli A., Straub P. W.* Fibrin polymerization studied by static and dynamic light-scattering as a function of fibrinopeptide A release. *Biopolymers*. 1982, 21(11), 2205–2223.
4. *Medved L, Weisel J. W.* The Story of the Fibrin(ogen) αC-Domains: Evolution of Our View on Their Structure and Interactions. *Thromb Haemost*. 2021 Dec 13. https://doi.org/10.1055/a-1719-5584
5. *Protopopova A. D., Litvinov R. I., Galanakis D. K., Nagaswami C., Barinov N. A., Mukhitov A. R., Klinov D. V., Weisel J. W.* Morphometric characterization of fibrinogen's αC regions and their role in fibrin self-assembly and molecular organization. *Nanoscale*. 2017, 9(36), 13707–13716.
6. *Kollman J. M., Pandi L., Sawaya M. R., Riley M., Doolittle R. F.* Crystal structure of human fibrinogen. *Biochemistry*. 2009, 48(18), 3877–3886.
7. *Litvinov R. I., Yakovlev S., Tsurupa G., Gorkun O. V., Medved L., Weisel J. W.* Direct evidence for specific interactions of the fibrinogen alphaC-domains with the central E region and with each other. *Biochemistry*. 2007, 46(31), 9133–9142.
8. *Poznyak T. A, Kolesnikova I. M., Makogonenko E. M.* Changes in the spatial orientation of αC-regions in the fibrinogen molecule during its transformation into polymeric fibrin. *Proc. NAS of Ukraine*. 2012, v.5, 163–168. (In Russian).
9. Yang Z., Kollman J. K., Pandi L., Doolittle R. F. Crystal structure of native chicken fibrinogen at 2.7 Å resolution. *Biochemistry*. 2001. 40(42), 12515–12523.
10. *Burton R. A., Tsurupa G., Medved L., Tjandra N.* Identification of an ordered compact structure within the recombinant bovine fibrinogen alphaC-domain fragment by NMR. *Biochemistry*. 2006, 45(7), 2257–2566.
11. *Varetska T. V.* Microheterogeneity of fibrinogen. Cryofibrinogen. *Ukr. Biokhim. Zhurn*. 1960, 32(1), 13–24. (In Ukrainian).
12. *Deutch D. G., Mertz E. T.* Plasminogen: purification from human plasma by affinity chromatography. *Science*. 1970, 170(3962), 1095–1096.
13. *Soloviev D. A, Ugarova T. P.* Isolation and characteristics of alpha-specific thrombin-like enzymes from venoms of the common pit viper [Agkistrodon halys halys] and the eastern pit viper [the central Asian subspecies Agkistrodon halys blomhoffii]. *Biochemistry*. 1993, 5(8), 1221–1233 (In Russian).
14. *Medved L. V., Novohatni V. V., Privalov P. L.* Isolation and investigation of structural organization of active and inactive forms of fibrinogen D-fragment molecule. *Mol. Biol. (Moskwa)* 1982, 16(6), 1195–1202. (In Russian).
15. *Marder V. J., Budzynski A. Z., Barlow G. H.* Comparison of the physicochemical properties of fragment D derivatives of fibrinogen and fragment D-D of cross-linked fibrin. *Biochim. Biophys. Acta*. 1976, 427(1), 1–14.
16. *Lugovskoy E. V., Gritsenko P. G., Kapustianenko L. G. Kolesnikova I. N., Chernishov V. I., Komisarenko S. V.* Functional role of Bbeta-chain N-terminal fragment in the fibrin polymerization process. *FEBS J*., 2007. 274, 4540–4549. https://doi.org/10.1111/j.1742-4658.2007.05983.x
17. *Belcher H. A., Litwa K., Guthold M., Hudson N. E.* The Applicability of Current Turbidimetric Approaches for Analyzing Fibrin Fibers and Other Filamentous Networks. *Biomolecules*. 2022, 12(6), 807. https://doi.org/10.3390/biom12060807
18. *Medved L. V., Gorkun O. V., Privalov P. L.* Structural organisation of C-terminal parts of fibrinogen Aα-chains. *FEBS Lett*. 1983, 160(1–2), 291–295.

ВПЛИВ D І DD ФРАГМЕНТІВ ФІБРИНУ НА ПОЛІМЕРИЗАЦІЮ ФІБРИНОГЕНУ ТА ЙОГО ФРАГМЕНТА Х, ЩО АКТИВУЮТЬСЯ ТРОМБІНОМ АБО АНЦИСТРОНОМ

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Мета. Досліджували роль комплексу між αC-регіоном та BβN-доменом, який формується після видалення FpA на молекулі фібрину desA, на початкових етапах полімеризації фібрину.

Методи. Для вивчення впливу D і DD фрагментів фібриногену на полімеризацію фібриногену і фрагменту фібриногену X було використано метод турбідиметрії, а також методи виділення, очищення та фрагментації фібриногену, мономерного та поперечно-прошитого фібрину, плазміногену.

Результати. Показано, що фрагмент DD повністю гальмував процес полімеризації у всіх досліджених системах («Fg + Thr», «Fg + Anc H», «X + Thr», «X + Anc H»). Фрагмент D інгібував полімеризацію фібрину на всіх стадіях у системі «Fg + Thr», але в системі «Fg + Anc H» майже не впливав на полімеризацію фібрину. В обох системах «X + Thr» і «X + Anc H» фрагмент D слабо пригнічував самозбирання молекул фібрину в протофібрили, але прискорював процес латеральної асоціації у другій системі.

Висновки. Отримані дані свідчать, що комплекс між αC-регіоном та BβN-доменом фібрину desA на початковій стадії полімеризації підтримує швидкість самозбирання та латеральної асоціації протофібрил фібрину desA, захищаючи його олігомери від деполімеризувального впливу фібриногену.

Ключові слова: фібриноген, фібрин, фрагмент DD, фрагмент Х, комплекс BβN-домен-αC-регіон.