Inhibition of Thrombin Activity with DNA-Aptamers

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 7, pp. 41-45, July, 2009
Original article submitted December 9, 2008

The effects of two DNA aptamers (oligonucleotides) 15TBA and 31TBA (15- and 31-mer thrombin-binding aptamers, respectively) on thrombin activity were studied. Both aptamers added to human plasma dose-dependently increased thrombin time (fibrin formation upon exposure to exogenous thrombin), prothrombin time (clotting activation by the extrinsic pathway), and activated partial thromboplastin time (clotting activation by the intrinsic pathway). At the same time, these aptamers did not modify amidolytic activity of thrombin evaluated by cleavage of synthetic chromogenic substrate. Aptamers also inhibited thrombin-induced human platelet aggregation. The inhibitory effects of 31TBA manifested at lower concentrations than those of 15TBA in all tests. These data indicate that the studied antithrombin DNA aptamers effectively suppress its two key reactions, fibrin formation and stimulation of platelet aggregation, without modifying active center of the thrombin molecule.

Key Words: *thrombin; DNA aptamers; fibrinogen; blood clotting; platelets*

Aptamers are single-stranded DNA or RNA oligonucleotides (up to several tens bases long) capable of specific binding to the target molecules. Due to their complex 3D structure, these oligonucleotides recognize various molecules, from small ligands to large proteins. Some aptamers can bind to their targets with high affinity and inhibit their biological activity. Primary selection of aptamers is realized from combinatorial library of random oligonucleotides (up to 10^{15} molecules) by the SELEX method (Systematic Evolution of Ligands by Exponential enrichment). The method includes several cycles of oligonucleotide selection by their binding with the studied molecule (usually immobilized on a solid carrier). Subsequent improvement of basal aptamers selected from the library (improvement of stability, affinity, *etc.*) can be realized by directed design, including computer simulation [1,5,11].

Thrombin is a multifunctional serine proteinase regulating many physiological processes (coagulation, anticoagulation, fibrinolysis, and platelet aggregation). The direction (specificity) of its action is determined by the involvement of at least two sites of thrombin molecule (enzyme active center and regions of specific "recognition" of many substrates and cofactors) in hydrolysis of high-molecular substrates. Thrombin not only causes the formation of fibrin, the structural base of clots, at the site of vessel wall injury, but also stimulates its own formation via activation of stimulating factors V, VIII, XI. Thrombin is most potent of the known inductors of platelet activation. It specifically reacts with PAR (protease activated receptors) on the platelet membrane and cleaves the peptide from the extracellular N-terminal of their amino acid sequence. The resultant new N-terminal fragment interacts with the special site of the receptor, which leads to platelet activation and subsequent aggregation [8].

Thrombin inhibitors are the first-line drugs for prevention and treatment of clotting abnormalities. The best known antithrombin agents are heparin and its low-molecular derivatives. They inhibit thrombin functions by stimulating antithrombin III, its main endogenous inhibitor. The drawbacks of heparin are its indirect action, binding to other plasma proteins

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0007-4888/09/1481-0033

and cells in the vascular network, and such untoward effects as thrombocytopenia and potentiation of platelet activation. Direct inhibitors of thrombin, such as hirudin (leach protein) and its derivatives (bivalirudin) and some low-molecular organic compounds (argatroban, melagatran) are free from these flaws. All of them modify thrombin active center (or active center together with substrate binding center) and nonselectively inhibit its pro- and anticoagulant effects [4]. Direct inhibitors of thrombin can be also created on the basis of aptamers [2,10]. Moreover, specific inhibition of procoagulant activity of thrombin can be also achieved with aptamers due to blockade of its bindings sites for fibrinogen and PAR.

We studied the effects of two thrombin-binding aptamers (TBA), 15TBA and 31TBA (15- and 31-mer DNA nucleotides), on thrombin-dependent coagulation reactions, amidolytic activity of thrombin, and thrombin-induced platelet aggregation. The sequence and antithrombin characteristics of 15TBA were described earlier [3,6]. The sequence of 31TBA is also known [7], but its effects on functional activity of thrombin were not studied.

MATERIALS AND METHODS

Aptamers 15TBA (GGTTGGTGTGGTTGG), 31TBA (CACTGGTAGGTTGGTGTGGTTGGGGCCAGTG), and control 31TBA aptamer with G6A substitution (31TBAG6A) were synthesized on an automated synthesizer Applied BioSystems 380b. Human thrombin with specific activity of 4422 U/mg protein (Haematologic Technologies Inc.) was used in the study. Thrombin receptor activating peptide (TRAP) with SFLLRN amino acid sequence was a kind gift from Dr. M. D. Ovchinnikov (Cardiology Research Center).

Thrombin time, prothrombin time, and activated partial thromboplastin time (APTT) were evaluated on an STA-Compact analyzer (Diagnostica Stago). The plasma was obtained by centrifugation (10 min, 30 rpm) of donor blood stabilized with 0.11 M sodium citrate ($^1/_{10}$ volume). Plasma (0.1 ml) and aptamer solution (0.1 ml) in 25 mM HEPES buffer (pH 7.4) with 150 mM NaCl and 5 mM KCl were put into a coagulometer cuvette and the mixture was incubated for 2 min at 37°C. For measurement of the thrombin time, the reaction was started by adding 0.1 ml thrombin (6 U/ml, in HEPES buffer with 1 mg/ml BSA), for measurement of the prothrombin time the reaction was started by adding 0.1 ml Ca-thromboplastin (STA Neoplastin Plus, Diagnostica Stago), and for APTT measurement the reaction was started by adding 0.1 ml STA APTT reagent (Diagnostica Stago) and (after 3 min) 0.1 ml 25 mM $CaCl_2$. The time of clot formation was evaluated in all tests. Thrombin amidolytic activity was evaluated on an FP-910 analyzer (Labsystems) by hydrolysis of the substrate (Tos-Gly-Pro-Arg-pNA; Chromozym TH, Boehringer Mannheim GmbH). Hydrolysis rate was evaluated by optical density increment at 405 nm/min (DA_{405}/min). Buffer (control) or aptamers (in a final concentration of 1 μM) were added to 150 μl thrombin solution (0.4 U/ml) in 0.1 M Tris-HCl buffer (pH 8.2) with 150 mM NaCl, 5 mM KCl, and 1 mg/ml BSA. After 5-min incubation at 37°C the reaction was started by adding 50 μl 2 mM chromogenic substrate.

Human platelets washed from the plasma were obtained as described previously [9]. Platelet aggregation was studied by light scatter in the platelet suspension (T %) in a BIOLA aggregometer (BIOLA). Aggregation was recorded at a platelet concentration of 3×10^8/ml at 37°C and mixing at 800 rpm for 4.5 min after thrombin addition. The aptamers were added to platelets 1-2 min before thrombin. Analysis of aggregation curves consisted in evaluation of the maximum aggregation level (T % max.).

RESULTS

Both aptamers added to human plasma dose-dependently prolonged thrombin time, prothrombin time, and APTT (Fig. 1, *a-c*). Thrombin time reflects the rate of fibrin clot formation after addition of exogenous thrombin to the plasma, while prothrombin time and APTT reflect the rate of clot formation under the effect of endogenous thrombin. Clotting is activated by the extrinsic pathway during measurement of the prothrombin time (activation of factors VII, X, V, and prothrombin in succession), while during measurement of APTT clotting is activated by the intrinsic pathway (successive activation of factors XII/prekallikrein, XI, IX, VIII, X, and prothrombin). Hence, both aptamers inhibited the formation of fibrin, stimulated by exogenous thrombin and by endogenous thrombin, formed as a result of activation of the plasma coagulation cascade. Aptamer 31TBA exhibited more pronounced inhibitory effect than 15TBA: similar effects were observed at lower concentrations of 31TBA. Differences in the effects of the test aptamers were more marked in clotting stimulation by endogenous thrombin (prothrombin time and APTT). Control aptamer with a single nucleotide substitution (31TBAG6A) in a concentration of 1 μM did not change fibrinogen clotting under the effect of exogenous (thrombin time 12.5 and 12.0 sec without and with 31TBAG6A, respectively; means of 2 experiments) and endogenous thrombin (APTT: 32.2 and 30.0 sec without and with 31TBAG6A, respectively; means of 2 experiments). Neither 15TBA, nor 31TBA modified thrombin amidolytic activity towards short chromogenic substrate

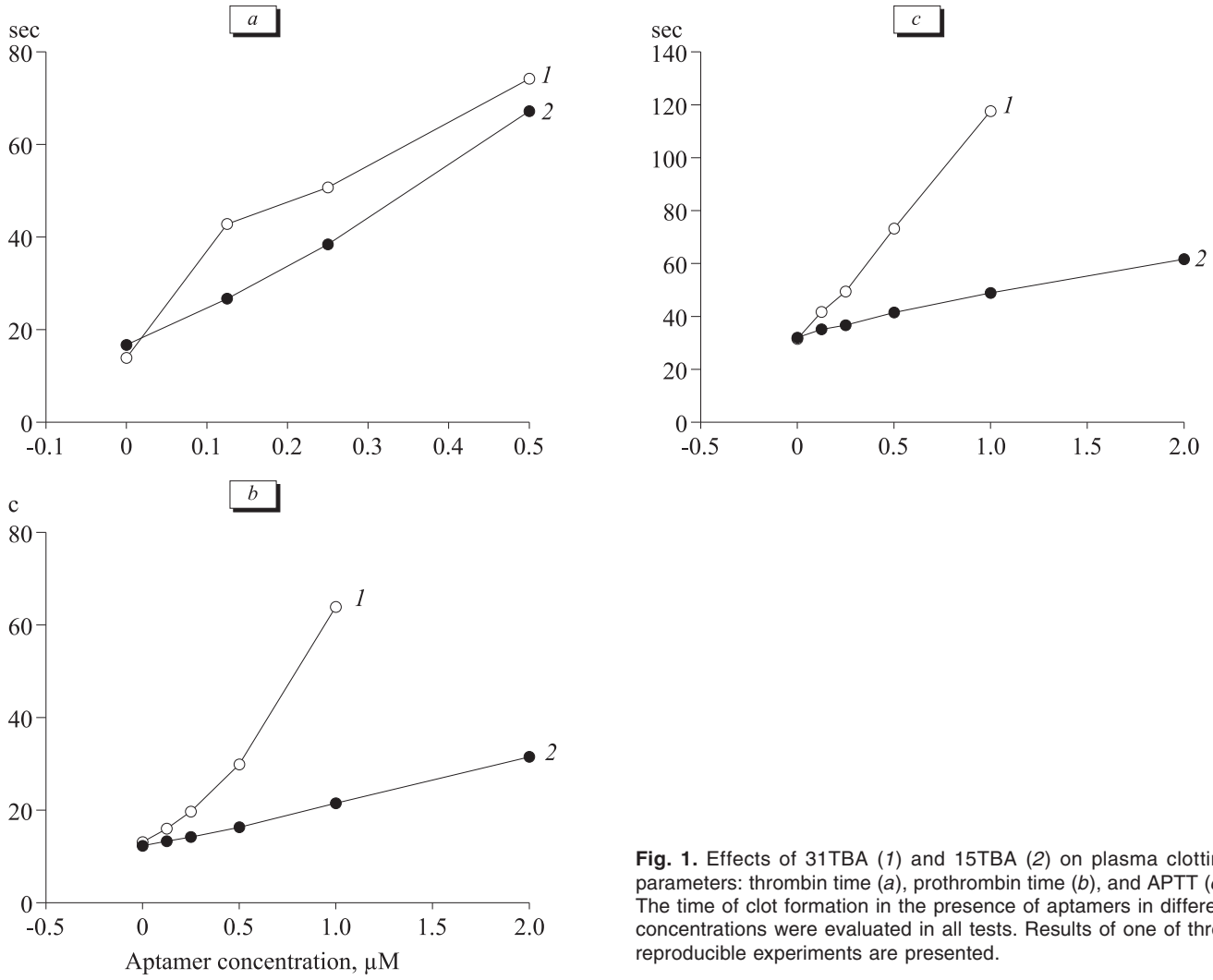

Fig. 1. Effects of 31TBA (*1*) and 15TBA (*2*) on plasma clotting parameters: thrombin time (*a*), prothrombin time (*b*), and APTT (*c*). The time of clot formation in the presence of aptamers in different concentrations were evaluated in all tests. Results of one of three reproducible experiments are presented.

Tos-Gly-Pro-Arg-pNA, reacting only with the active center. The degree of its cleavage recorded by optical density increment at 405 nm did not decrease in the presence of both aptamers. Amidolytic activity of thrombin was 0.259 in the presence of 15TBA, 0.273 in the presence of 31TBA, and 0.257 DA_{405}/min in the presence of control 31TBAG6A aptamer (means of 2 experiments).

Aptamers 15TBA and 31TBA effectively inhibited thrombin-induced aggregation of platelets washed from the plasma (Fig. 2). Control 31TBAG6A aptamer did not reduce the level of aggregation (Fig. 2, *a*). Aptamers did not modify TRAP-induced (10 μM) aggregation (the effect of TRAP on PAR requires no proteolytic cleavage of its N-terminal domain), the maximum aggregation being 43.1 T % and 45.3 T % without and with 1 μM 31TBA, respectively (the means of 2 experiments). Aptamer 15TBA inhibited platelet aggregation in higher concentrations than 31TBA (Fig. 2, *b*). Complete blocking of aggregation stimulated with 0.1 U/ml thrombin was generally observed in different experiments with 15TBA in concentrations of at least 0.2 μM and 31TBA in a concentration of about 0.1 μM.

Aptamer 15TBA is the first antithrombin aptamer and its characteristics are well studied [3,6]. We confirmed that it inhibits thrombin clotting and thrombin stimulation of platelet aggregation. The other aptamer, 31TBA, is based on the 15TBA aptamer sequence supplemented with 8 nucleotides from the 3' and 5' terminals [7]. Its antithrombin effects were not studied until present. We showned by computer simulation that this aptamer has a large area of contact with thrombin and therefore exhibits high affinity for thrombin: the thrombin-31TBA complex dissociation constants are more than 2-fold lower than of thrombin-15TBA complex (data not presented). Our studies showed that 31TBA more effectively inhibits the functional activities of thrombin in comparison with the basic 15TBA aptamer.

Both aptamers inhibited thrombin-induced formation of fibrin and platelet aggregation without modify-

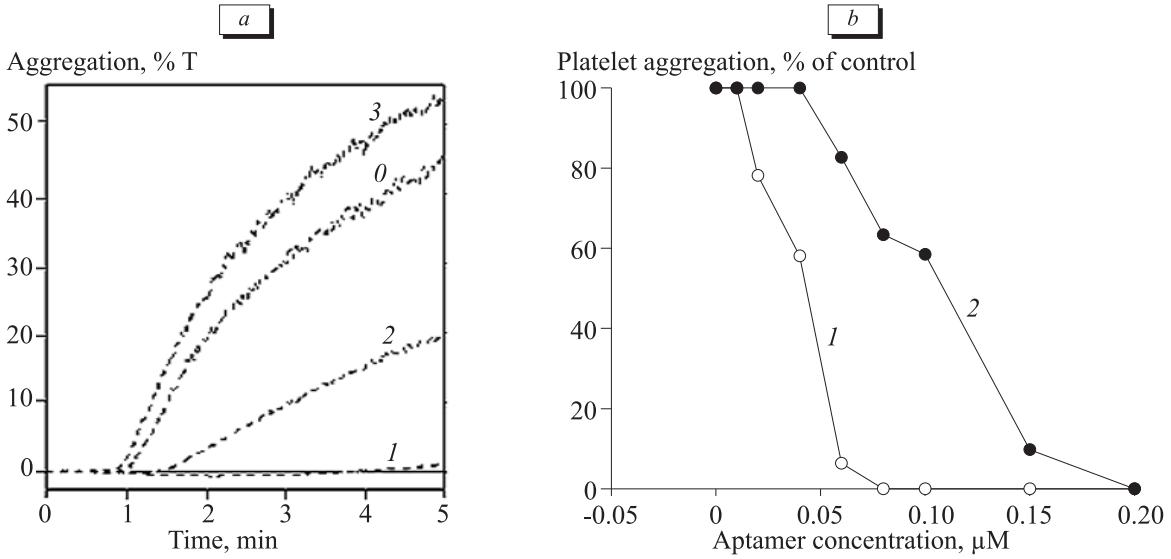

Fig. 2. Inhibition of thrombin-induced platelet aggregation with 15TBA and 31TBA. *a*) without aptamers (*0*); 0.1 μM 31TBA (*1*); 0.1 μM 15TBA (*2*); 1 μM 31 TBAG6A (*3*). Platelet aggregation stimulated with 0.1 U/ml thrombin. *b*: platelet aggregation stimulated with 0.1 U/ml thrombin without aptamers (100%) and in the presence of different concentrations of 31TBA (*1*) and 15TBA (*2*). Maximum level of aggregation evaluated in % of control. Results of one of three reproducible experiments are presented.

ing amidolytic activity of the enzyme (without blocking its active center). These results suggest that they most likely bind exosite I in thrombin molecule playing the key role in recognition and high-affinity reaction of thrombin with fibrinogen and platelet PAR.

The study was supported by Federal Agency for Science and Innovations (State Contract No. 02.51211.2242) and Russian Foundation for Basic Research (grant No. 08-04-01244-a).

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