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REVIEW

Molecular Mechanisms of Thrombosis. Fundamental and Applied Aspects of the Contact Activation

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Abstract—The human hemostatic system stops bleeding when vascular lesions occur. This is one of the most vulnerable physiological systems. Hemostatic disorders, which are encountered in patients with various diseases (i.e., atherosclerosis, trauma, sepsis, and cancer), may lead to both hemorrhage and thrombosis. Pathological clot formation is dangerous, and thrombosis is the most common cause of death in developed countries. Activated factor XII (fXIIa), a key enzyme of the contact pathway of coagulation, does not play a significant role in maintaining hemostasis but is involved in the pathological blood clotting. Therefore, this enzyme is a promising target for the development of antithrombotic drugs. Inhibition of the contact pathway is essential for any ex vivo manipulation involving blood (i.e., transfusion, cardiopulmonary bypass, and blood sampling) and when blood is exposed to foreign surfaces. In this review we consider modern views on the contact activation and the pathophysiological role of this process in the blood coagulation and other systems of the organism. Existing inhibitors of the contact pathway exhibit two mechanisms of action: a competitive binding to the active site of fXIIa and an allosteric inhibition via the N-terminal regulatory domain, which blocks activation by anionic surfaces and binding to physiological substrates. These two mechanisms are discussed. A comparison of existing inhibitors, with special emphasis on the most promising ones, and the criteria for new fXIIa inhibitors are also provided.

Keywords: hemostasis, coagulation factor XII (Hageman factor) inhibitors, contact activation, thrombosis, clinical diagnostics

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INTRODUCTION

Thrombosis, i.e., blood clotting inside a blood vessel, is a reason of severe diseases with high mortality rate [1]. Thrombus can occlude the vascular lumen at the site of its formation or cause a thromboembolism downstream the blood flow. The vessel occlusion causes a disturbed oxygenation of the surrounding tissue (hypoxia or anoxia), leading to the infarction and tissue necrosis [2]. Some of the most dangerous thrombosis manifestations are pulmonary vein embolism, ischemic stroke and myocardial infarction. Despite the high incidence rate of a pathological thrombus formation, an efficient and safe method of thrombosis prophylaxis and treatment, which does not repress hemostasis, remains unknown [3]. The necessary condition of a thrombus formation is the hyper coagulant state of the blood clotting system, which in normal state is responsible for the bleeding stop at wounding of the vascular bed [4, 5]. The blood coagulation consists of vascular, platelet, and plasma chains of hemostasis; the latter is an enzymatic cascade of the coagulation factor activation resulting in the formation of a fibrin clot [6]. In the present review we consider in detail the contact pathway of coagulation, since a hypothesis about the role of contact activation in the pathological thrombus formation was confirmed recently [7, 8]. Further we consider its molecular mechanisms.

1. CONTACT ACTIVATION OF BLOOD COAGULATION

1.1. Activation of Factor XII

Contact activation is initiated by the auto-activating coagulation factor XII (fXII, or Hageman factor), which binds to negatively-charged (foreign) surfaces in a complex with the high-molecular weight kininogen (HMWK) and plasma pre-kallikrein (PK) [9, 10]. HMWK includes a positively-charged histidine-rich domain, this domain and zinc ions mediate binding to the surface [11, 12]. Circulating blood plasma contains approximately 0.3 μM of fXII, which is a single-chain polypeptide of 80 kDa containing N-terminal regulatory domains (52 kDa) and C-terminal catalytic domain (28 kDa). The catalytic domain is not active in a circulating factor; it turns into a surface-bound active enzyme when it is (auto)activated via a point proteolysis. The result is a trace amount of catalyti-

cally active alpha-fXIIa, which consists of disulfide-bound heavy and light chains (52 and 28 kDa, respectively). Alpha-fXIIa containing the surface-binding heavy chain may activate PK into kallikrein. In its turn, kallikrein catalyzes the proteolytic activation of alpha-fXIIa, and further – activation of beta-fXIIa, a 28 kDa catalytic domain. Beta-fXIIa, as well as alpha-fXIIa, activates kallikrein. However, alpha-fXIIa is a major factor in the contact-activated coagulation, while the other forms of fXII are not able to bind to the foreign surfaces and activate factor XI [10]. Mutual activation of fXIIa and kallikrein results in an exponential generation of both of these proteases, which is regulated by endogenous plasma inhibitors (SERPINs, e.g. Cl-inhibitor). These plasma inhibitors form a stable covalently bound intermediate with the active proteases and block their active site [13, 14].

1.2. Physiological Role of fXII

During the contact activation the activated factor XI (fXIa) is generated; it triggers the coagulation cascade resulting in sequential activation of factors IX, X and thrombin [15, 16]. Thrombin converts fibrinogen, a soluble plasma protein, into fibrin and activates fXIII; fibrin self-polymerizes, whereas fXIIIa covalently links the fibrils of fibrin into a spatial network, representing a framework of a gelatinous clot [17]. However, the contact-activated clotting seems to be not physiologically relevant in normal hemostasis. The physiological process of the coagulation cascade activation and clot formation (e.g., at wounding of blood vessel) is believed to occur by another way: via contact of blood with tissue factor (TF, or thromboplastin), a transmembrane glycoprotein found in all tissues except for blood vessel endothelium. This contact results in binding of factor VII (fVII) of blood plasma with its cofactor, TF, followed by fVII activation and formation of the extrinsic tenase complex (complex between cofactor TF and enzyme fVIIa), which activates factors IX and X and initiates the clot formation at the site of vessel injury [6, 18].

In addition, fVII can be activated by alpha- and beta-forms of fXIIa [19–21]. This reaction is inhibited by C1-inhibitor at the physiological temperature, but the inhibitor loses its activity near 0°C. Therefore, at low temperature the effect of the "cold" activation arises. This effect should be taken into account when the frozen plasma samples are used in the coagulation assaying. Thawing of a frozen sample prior to testing may result in the cold activation and unwanted background activation of the coagulation cascade.

Alpha-fXIIa can modify a structure of the fibrin clot independently on its pro-coagulant activity. If the heavy chain of alpha-fXIIa is bound to fibrin and the light chain is proteolytically active, a dense fibrinolysis-resistant clot is formed [22]. However, the physiological role of its phenomenon remains unknown. It is shown that the anionic surface-mediated fXIIa generation can be inhibited with the beta-subunit of fXIII, generated as a result of the activation with physiological concentrations of thrombin [23].

Alpha- and beta-fXIIa, as well as kallikrein, are able to activate plasminogen, resulting in a formation of plasmin, a key enzyme of the fibrinolysis system, which proteolytically degrades fibrin fibrils in a clot [24]. Moreover, fXII and alpha-fXIIa can bind to the epidermal growth factor receptor (EGFR) via their N-terminal domain; and this binding is independent on their catalytic activity [12]. This association causes the receptor activation and stimulates the growth of the endothelial cells. Therefore, fXII participates in angiogenesis, a process of vascular development.

Any surfaces foreign for an organism may absorb fXII during the contact with blood and lead to the contact activation. The contact activation occurs in vitro during the blood collection, via the walls of plastic or glass tubes, or ex vivo during the catheterization, stent procedure and usage of the cardio-pulmonary bypass [25, 26]. Factor XII auto-activation in vivo is shown to be mediated also by the surfaces of activated platelets and bacterial cells, as well as by collagen or polyphosphates (including extracellular RNA and DNA), i.e., by the substances which are in abundance near the site of the vessel injury [27–29].

A physiological role of the contact activation is still debated, beginning from its discovery in 1955, when Ratnoff and Colopy noted the absence of glass-mediated clotting in the plasma sample of their patient, Mr. John Hageman [30]. The patient lacked a protein, which now is called fXII. Despite the fXII deficit, he had normal hemostasis. Moreover, it was observed later that the fXII deficiency was not associated with bleedings [7, 31].

An attempt to clarify our understanding of fXII physiological role was made in some evolutionary works. Comparing the genomes of Chordata clades, it was shown that fXII gene was present in *Chordata*, making its first appearance in amphibians (*Amphibia*) and being lost in birds (*Aves*). Also, fXII protein is absent in marine mammals, such as whales, dolphins, etc. (Cetacea). When the amphibians have evolved, an ortholog of both plasma pre-kallikrein and fXI has first made its appearance. The genome duplication, which gave rise to the distinct genes of pre-kallikrein and fXI, resulted, presumably, in appearance of pouched marsupials (*Marsupialia*) [32–34]. These results imply that the contact pathway would be beneficial for the evolution of terrestrial vertebrates.

1.3. Pathological Significance of the Contact Activation

While the role of fXII in normal hemostasis is still unclear, fXII is proposed to be involved in the pathological thrombus formation. The hypothesis was supported in various animal models, including mice, rat and rabbit models of $FeCl_3$-induced thrombosis [7, 8,

35], acute ischemic stroke [36], collagen-induced thromboembolism of pulmonary artery and in the aorta ligation model [7, 37]. Factor XII knock-out mice, as well as the wild-type mice with infused selective inhibitors of fXII, exhibited a reduced thrombus weight, a reduced volume of the damaged tissue, and a reduced mortality, as compared to animals with a normal level of fXII. When a normal level of fXII was restored in fXII-deficient mice, the extensive thrombosis was developed. However, deficiency of fXII did not lead to deficits of hemostasis, as it was shown in the tail and cuticle bleeding models [7, 8, 36]. Therefore, fXII does not play a significant role in hemostasis but it participates in the pathological thrombus formation in hypercoagulant states [29].

Factor XIIa comprises a promising target for the new antithrombotic therapeutics [29, 38, 39]. A major disadvantage of the currently used anticoagulants is a risk of bleedings, which in the case of internal bleedings can be life-threatening [40–42]. These anticoagulants (such as heparin, rivaroxaban, dabigatran, etc.) inhibit key enzymes of the coagulation cascade (such as thrombin and fXa) and compromise both thrombosis and hemostasis [43]. Development of the therapeutics based on the selective inhibitors of fXIIa would allow us to achieve a new approach in the thrombosis treatment which would exclude any bleeding risk [39].

1.4. Modern Assays of Hemostasis. Impact of the Contact Activation on Global Coagulation Assaying

Global assays of hemostasis are very useful in the investigation of the coagulation cascade and in the diagnostics of hemostasis deficits caused by various pathologies. In these assays coagulation is initiated in vitro by some activator (e.g., tissue factor), thus reflecting a physiological process of blood clotting in vivo [44–46]. Currently, the most popular assays are thrombin generation assay (TGA), thromboelastography (TEG) and thrombodynamics (TD) assays [47]. When thrombin generation or thromboelastography are assayed, coagulation is activated by mixing blood or plasma with TF, followed by measurements of the cleavage rate of the thrombin-specific fluorogenic substrate (TGA) or the plasma visco-elasticity during clot formation and lysis. Both measurements result in kinetic curves of the coagulation process which are described by several parameters, such as lag-time (delay between activation moment and the start of coagulation), maximal amplitude (of thrombin concentration or blood viscosity) and time to the maximum, maximal slope of the curve, etc. During assaying of TD coagulation is initiated with a surface with adsorbed TF, and both thrombin and fibrin clot propagation from this surface are investigated [48]. Herein, coagulation is described by the lag-time parameter and by the rates of fibrin clot growth into a bulk of plasma.

The tests described above allow discovering of a pathological state of hemostasis in patients by a deviation of the tests' parameters from a normal range typical for healthy donors. When low, near physiological concentrations of TF are used, the assay parameters may depend on the concentrations of coagulation factors. For example, in patients with hemophilia A, B and C (deficiency in factors VIII, IX and XI, respectively) the lag-time is increased and the thrombin peak height and the clot growth rate are decreased [46, 49]. In hypercoagulant states with an increased risk of thrombosis, opposite effects are observed [45, 50]. Moreover, the TD assay in a hypercoagulant plasma sample results in TF-independent growth of spontaneous fibrin clots far from the surface with TF. The global hemostasis assays with low TF concentrations are sensitive to the deviations from the normal coagulation and last from 30 min to 1 h and more.

As described above, clotting can be activated by the contact pathway in vitro by almost every foreign surface contacting with blood: a needle, tube walls, etc. [25, 26]. This effect is a serious obstacle for blood storage, preparation of its components and the coagulation assays. During assaying of the global hemostasis, plasma is preliminary prepared (the preparation includes thawing of a frozen sample); during the preparation the contact activation occurs and the generated fXIIa initiates TF-independently the formation of a fibrin clot 15–30 min after both re-calcification (i.e., restoration of the physiological concentration of calcium ions) and the assay start. Simultaneously, fXIIa-mediated cold activation of fVII increases the background activation of coagulation in a bulk of plasma and complicates the assaying of frozen plasma samples. As a result, TF-triggered clotting is distorted by the artificial fXIIa-triggered coagulation [51, 52]. For example, when the TD is assayed in a plasma sample from a healthy donor, spontaneous clots similar to those found in the hypercoagulant plasma can appear, thus resulting in a decreased assay sensitivity to hypercoagulation. It was shown that if fXIIa was blocked with a selective inhibitor during a plasma preparation step, spontaneous clots grew only in the plasma sample from a patient with hypercoagulation and never in healthy human plasma [50, 53]. Similarly, when the thrombin generation or thromboelastography are assayed, contact activation of the sample increases the value of maximal amplitude and decreases the coagulation times in healthy plasma, comparing to the healthy plasma with preliminary blocked contact activation [52, 54, 55]. In summary, a correct assay of the global hemostasis utilizing low TF concentrations demands that the contact activation should be preliminary blocked with a highly effective and selective inhibitor.

1.5. Contact Activation in Surgery

Activation of clotting when blood contacts the foreign surfaces is an important issue during blood transfusion, catheterization, cardio-pulmonary bypass usage, etc. [25]. For example, a thrombus formed on the central vein catheter can occlude the blood flow or cause a pulmonary embolism [56]. The contact pathway activation is also responsible for the increased risk of thrombosis after autotransfusion [57]. To prevent clotting in the apparatus of cardio-pulmonary bypass, its tubes and other surfaces are covered with fXa or thrombin inhibitors, such as heparin, benzamidine, etc. [58, 59]. A shortcoming of this method is that the addition of fXa and thrombin inhibitors does not prevent the generation of fXIIa, which may trigger coagulation after autotranfusion into the patient's vascular bed.

A physiological role of fXII remains unclear, with some general hypotheses about fXII as a linkage between the systems of plasma and platelet hemostasis, complement, fibrinolysis and vasodilation [38, 60, 61]. The existing knowledge about fXII elucidates rather its negative sides: it promotes thrombosis in blood vessels, complicates any manipulation with blood during transfusion and interferes with the highly sensitive hemostasis assays. A solution of these problems requires a highly selective fXIIa inhibitor, neutral to other coagulation factors. Further we consider and analyze the current methods of prevention of the contact activation, emphasizing the competitive inhibitors of fXIIa.

2. INHIBITORS OF THE CONTACT ACTIVATION

2.1. Non-Specific Inhibition of the Plasma Coagulation

The most common way to prevent contact activation of the plasma coagulation during blood sampling is a usage of collection tubes pre-filled with a calcium chelating agent (e.g., sodium citrate, EDTA, etc.) into a collection tube. Chelation of calcium ions blocks membrane-dependent reactions of fX and thrombin activation [18]. Prior testing the recalcification of plasma is performed. An excess of calcium ions is added to the sample in order to restore the concentration of free ions to the physiological value of 2 mM. The main disadvantage of chelation is that it does not block production of fXIa via fXI activation by fXIIa [155]. It was shown [62] that chelation and subsequent re-calcification change the dynamics of the fibrin clot formation, thrombin generation, and platelet aggregation. An alternative approach to prevent contact activation of the plasma coagulation is an application of a selective fXIIa inhibitor instead of a calcium chelating agent [52]. This makes it possible to avoid the unwanted activation of fXI.

2.2. Specific Inhibitors of fXIIa

2.2.1. Inhibitors of fXIIa from human plasma. There are a number of fXIIa inhibitors in human plasma: C1-inhibitor (C1INH) [14, 63], antithrombin-III (AT-III), α2-antithrombin, α2-macroglobulin [64], and histidine-rich glycoprotein [65, 66]. C1INH is a serpin, belonging to the family I04.001 of the α-1-protease inhibitors [67]. Until recently [68] C1INH was considered as the main plasma inhibitor of fXIIa. C1INH irreversibly inhibits fXIIa via a common mechanism for serpins. N-terminal loop of this inhibitor enters the active site of the protease. The subsequent cleavage of the peptide bond in the inhibitor leads to a rapid conformational change of serpin. As a result, the catalytic site of the protease is disrupted and the hydrolysis of the acyl-enzyme intermediate does not occur. Thus, serpin gets covalently bound to the protease [69]. However, it was shown that fXIIa—C1INH complexes were mainly produced in plasma after a surface-mediated activation (with kaolin, glass, etc.) [68], whereas in the case of fXIIa activation by activated platelets, the fXIIa—antithrombin complexes were predominant. Binding to the endothelial cells also protects fXIIa from inactivation by C1INH [70]. Thus, C1-inhibitor is not the major regulator of the contact pathway in the case of physiological activation of fXIIa.

Antithrombin-III (AT-III) belongs to the same family of α-1-protease inhibitors (I04.001) [67] as C1-inhibitor. This serpin inhibits fXIIa in the presence of its cofactor heparin [13]. AT-III binds to heparin at specific antithrombin-binding sites [71]. In the absence of AT-III, heparin activates fXIIa [72], because it is a negatively charged sulfated glycosaminoglycan.

Histidine-rich glycoprotein (HRG) suppresses contact activation [65] and also binds to heme [73], heparin, heparan sulfate [74], thrombospondin [75], plasminogen [76], and divalent metal ions [77]. HRG binds to alpha-fXIIa exosite. This has no effect on the alpha-fXIIa catalytic activity towards the chromogenic substrate but prevents interactions with macromolecular substrates such as fXII and fXI [66]. Mechanism of fXIIa inhibition by HRG is not completely understood yet. Presumably, histidine-rich glycoprotein binds to fXIIa and heparan sulfate [12, 75] via its N-terminal cystatin-domain, since Zn^{2+} ions improve binding of HRG to fXIIa by three orders of magnitude, and HRG loses its ability to inhibit fXIIa in the presence of heparin [66].

2.2.2. Protein fXIIa inhibitors from natural sources. Factor XIIa belongs to serine enteropeptidase family S1. This family includes trypsin-like proteases. Such proteases are widespread in nature. So, along with a number of plasma fXIIa inhibitors, there are a number of other inhibitors of the bacterial (ecotin) [79], plant (including inhibitors from seeds), or animal (blood-sucking insects) origin [80–85]. The majority of these

inhibitors is non-specific and also inhibits fXIa, fXa, fIXa, and thrombin. Nowadays (April, 2014), 12 protein non-plasma inhibitors of fXIIa are registered in the MEROPS database [67]. These inhibitors are relatively small proteins with molecular weight from 3 to 16 kDa [80–85] belonging to five different families. The most selective fXIIa inhibitors are corn trypsin inhibitor (CTI) and infestin 4 with inhibition constants of 2.5 nM [86] and 78 pM [81], respectively. Infestin-4 is a fourth domain of infestin from a stomach of the bloodsucking insect *Triatoma infestans*. Both inhibitors are canonical and interact with fXIIa via the standard mechanism [87]. Canonical inhibitors are characterized by protruding the protease-binding loop of a typical conformation [88, 89]. Such a loop is normally closed into a circle by a disulfide bridge [90], and in rare cases by strong non-covalent interactions [91]. This loop is cleaved in the active site of the protease in a substrate-like manner, however, the hydrolysis does not lead to the loss of the inhibitory activity: both forms of the inhibitor (such as single-stranded and double-stranded) retain their activity [87].

CTI is widely used to inhibit the contact activation in blood or plasma samples in TF-initiated coagulation assays, for example, in the thrombin generation assay [51, 52, 55, 92] and also for the surface modification of implants [59]. CTI belongs to the I6 family of cereal serine protease inhibitors [67]. Members of this family are bifunctional (these inhibitors suppress both serine protease and alpha-amylase activity) seed storage proteins. The latter suggests that such inhibitors evolutionarily emerged as a component of the system of seed protection from pests and diseases. This hypothesis is confirmed by the correlation of the high content of CTI in corn with the resistance to a number of fungal infections [93]. Although the CTI inhibition constant for fXIIa is in a nanomolar range, it has a number of disadvantages, in particular, CTI delays the onset of fibrinolysis, presumably by inhibiting tissue plasminogen activator [54]; moreover, it inhibits fXIa.

The most selective inhibitors of contact activation are likely to be found in organisms that feed on blood, because they need to keep it in a liquid state after eating. Infestin-4 is an example of such inhibitors [67]. This inhibitor belongs to the Kazal inhibitor family I1 and is one of the infestin domains. Infestin is a multidomain inhibitor found in the stomach of bloodsucking insect *Triatoma infestans*. Infestin prevents blood clotting by inhibiting thrombin, fXIIa, fXa and plasmin (K_i, 0.8 nM, 78 pM, 59.2 nM, and 1.1 nM, respectively) [81]. In 2010 infestin-4 was reported to be a selective fXIIa inhibitor [8]. It blocks fXIIa activity in the in vivo thrombosis models and prevents embolism without affecting any other parameters of hemostasis. Nevertheless, infestin-4 also exhibits inhibitory activity towards fXa ($K_i = 53$ nM) [8]. This activity is not desirable for the clinical or diagnostic use. However, this drawback has been overcome [94] by point amino acid substitutions in the loop interacting with the active site of fXIIa.

Recently two monoclonal antibodies 15H8 [35] and 3F7 [95] were developed for the selective fXIIa inhibition. These inhibitors reduced amount of platelets and fibrin in the thrombus in arteriovenous shunt model in primates and prevented thrombus formation in the $FeCl_3$-induced model of thrombosis in mice. Application of 3F7 instead of heparin prevents clot formation in the cardiopulmonary bypass and eliminates the risk of bleeding arising from the use of heparin. More sophisticated and potentially safe method of preventing thrombosis has been proposed previously when using antibody 14E11 [36] which blocked the fXIIa-mediated activation of fXI, leading to a 2-fold decreased brain volume affected in a model of acute ischemic stroke in mice.

However, despite the high selectivity of the inhibition of contact activation that is achieved with antibodies, their usage as a safe drug for thrombosis treatment is limited by the reasons common to all protein drugs: a need for frequent intravenous infusions, special requirements for their storage, and possible immune response [96]. Modern antithrombotic drugs are low molecular weight inhibitors of fXa or thrombin and do not possess these limitations, but their usage is associated with a risk of hemorrhages [40].

2.2.3. Artificial inhibitors. Selective binding to and tight inhibition of fXII can be achieved not only with natural proteinaceous inhibitors (predominantly, with competitive inhibitors) or specific antibodies. Development of selective DNA- or RNA-based inhibitors is a well-known method. In [97] aptamer R4cXII-1 was selected from the library of 10^{14} sequences; this RNA aptamer selectively binds to fXIIa with dissociation constant $K_d = 0.5$ nM and dose-dependently inhibits contact-activated coagulation with effective concentration IC_{50} approximately 0.3 μM. Noteworthy, R4cXII-1 does not affect fXIIa catalytic activity toward a low-molecular-weight chromogenic substrate or its physiological substrate pre-kallikrein but inhibits fXIIa activity toward another physiological substrate fXI with K_i approximately 1 nM. The aptamer blocks fXII auto-activation initiated with dextran sulfate or glass, and partially with kaolin and ellagic acid. The aptamer is proposed to bind to the fXIIa heavy regulatory chain in the site of binding of fXI and anionic surfaces, far from the pre-kallikrein binding site.

A new fXIIa inhibitor can be also developed by a peptide phage display technique. A library of cyclic peptides up to 10 amino acids is often used. For example, the new bicyclic peptide FXII402 inhibiting fXIIa with K_i of 1 μM [98], was selected by phage display of Xaa–Cys–$(Xaa)_4$–Cys–$(Xaa)_4$–Cys–Xaa peptides library (where Xaa was a varying aminoacid) against beta-fXIIa. Other coagulation-related proteases were not inhibited by the peptide concentrations up to 100 μM. Noteworthy, the peptide selection against

K_i for fXIIa inhibitors presented in the MEROPS database

Inhibitor	K_i	Reference
Corn trypsin inhibitor (CTI)	1 nM	[100]
Infestin-4	78 pM	[81]
Aprotinin	6.8 μM	[82]
Kallikrein inhibitor (*Oxyuranus* sp.)	2.38 μM	[83]
LITI (from *Leucaena leucocephala*)	n.d.	[84]
MCTI-1 (from *Momordica charantia*)	13 nM	[35]
CMTI-III (from *Cucurbita maxima*)	70 nM	[85]
MCTI-II (from *Momordica charantia*)	56 nM	[85]
LCTI (from *Luffa cylindrica*)	3.8 nM	[85]
MCTI-III (from *Momordica charantia*)	1.6 μM	[85]
mAb 15H8	n.d.	[35]
mAb 3F7	n.d.	[95]
mAb 14E11	n.d.	[36]
Aptamer R4cXII-1	1 nM	[97]
Bicyclic peptide FXII402	1 μM	[98]

mAb, monoclonal antibody; n.d., not determined.

alpha-fXIIa and/or the library Xaa–Cys–$(Xaa)_6$–Cys–$(Xaa)_6$–Cys–Xaa yielded only non-active peptides, which presumably bound to the heavy chain of alpha-fXIIa. Due to its low inhibitory activity, FXII402 alone could not be used for the anticipated applications, but Baeriswyl et al. also developed a bicyclic kallikrein inhibitor PK128 with K_i 0.3 nM [99]. PK128 together with FXII402 were active in plasma leading to a 2–3-fold elongation of the activated partial thromboplastin time (aPTT) at concentrations of 50 μM.

Another method of selective repression of the contact activation is an application of antisense oligonucleotides (ASO), which block mature fXII expression in hepatocytes (the approach resembles gene therapy). Anti-fXII ASO reduce fXII plasma level greater than 90% and double the occlusion time of jugular vein catheter in rabbit in comparison with a control or anti-fVII ASO [37]. An obvious drawback of this method is a complete elimination of fXII from the body, leading to disruption of all physiological functions of fXII, including production of kallikrein which in its turn activates the systems of fibrinolysis and complement, as well as the bradykinin release.

3. COMPARISON OF THE EXISTING INHIBITORS. APPROACHES TO THE DEVELOPMENT OF NOVEL INHIBITORS

The inhibitory constants for the above-described fXIIa inhibitors are shown in the table. Most of the discussed inhibitors are proteins and act via the competitive mechanism [87]. Aside from the obvious difficulties associated with the infusion of a protein drug into a patient's circulatory system, protein inhibitors have two major drawbacks. First, the use of inhibitors with K_i more than 10 nM is inexpedient, since effective concentrations of such inhibitors in plasma is about 50 μM or more [85, 98]. Another problem is an insufficient selectivity because even those inhibitors with $K_i \approx 1$ nM or less have a typical effective plasma concentration in the range of 1–10 μM. A significant difference between K_i values in a buffer containing chromogenic low-molecular weight substrate, and an effective inhibitory concentration in plasma is caused by (1) a relatively high normal level of fXII in plasma (approx. 0.3 μM) and (2) a presence of plasma physiological fXIIa substrates which compete with an inhibitor for binding to the protease. Nevertheless, plasma albumin does not bind to the protein inhibitors and does not cause any increase in the effective inhibitory concentration (unpublished data). Therefore, the contact activation inhibitor should possess selectivity toward fXIIa against other paralogous coagulation factors more than 10^4. Currently, natural fXIIa inhibitors do not possess a high selectivity [94, 100].

A high degree of selectivity can be achieved with specific antibodies to fXIIa, including antibodies that bind to individual domains of the factor and selectively block some of its functions [36]. However, the practical use of antibodies is often difficult due to the need for direct intravenous infusions and the specific storage requirements [96]. More promising agents are aptamers similar to R4cXII-1 [97]. R4cXII-1 binds to N-terminal domain but not to the catalytic domain of fXII(a), thereby blocking the binding of the factor to anionic surfaces which promote its auto-activation and binding with fXI [101]. An advantage of aptameric therapeutics is a possibility to develop an antidote, representing a complimentary single-chain oligonucleotide [102]. Of course, further studies are needed to confirm the benefits of this type of drugs.

If the crystal structure of a target protease is known, the quantitative approaches such as docking and molecular dynamics can be applied for development of the selective low molecular weight inhibitors [103]. This method is carried out for initial screening of ligands that are most closely associated with the active site of the enzyme. Then the selected ligands are synthesized and their inhibitory activity is verified in the in vitro tests. Unfortunately, in the case of fXIIa this strategy is not applicable, because the spatial structure of the catalytic domain is unknown. As a result, there is no selective synthetic inhibitors, effectively blocking fXIIa activity in plasma, available unlike fXa, thrombin and other coagulation factors [104, 105]. That is why screening the aptamer or phage libraries seems more promising in the case of fXIIa [97, 98].

4. REQUIREMENTS TO AN INHIBITOR OF THE CONTACT ACTIVATION

New inhibitors of the contact activation should meet a number of criteria. For instance, the minimum requirement for a contact activation inhibitor, employed in the diagnostics, is a high selectivity towards fXIIa. Repression of the contact pathway should not affect the TF-initiated formation of a fibrin clot [106].

Successful inhibition of fXIIa in vivo as a part of the antithrombotic therapy should be also accompanied by the minimized effect on other components of the coagulation system, fibrinolysis, kinin-kallikrein system, and inflammation. Prevention of fXII (auto)activation seems to be one of the most perspective approaches. This prevention can be achieved via different ways: via direct blocking of Arg353–Val354 bond hydrolysis and subsequent conversion into active alpha-fXII; prevention of auto-activation mediated by anionic surfaces; elimination of Zn^{2+} ions [11], polyphosphates [27], and nucleic acids [28]. Application of positively charged peptides, competing with fXIIa for anionic surfaces, is a new promising approach of inhibition of contact activation. Use of the direct fXIIa inhibitors seems to be less promising, since this type of inhibitors can interfere with other physiological systems involving fXIIa [107].

CONCLUSIONS

In this review we consider the molecular mechanisms of fXII (auto)activation and its effects on blood coagulation via activation of the intrinsic pathway and fVII ("cold activation"), as well as its roles in the fibrinolysis activation and angiogenesis. Nevertheless, the physiological role of fXII is not currently clear, however, fXII is proposed to become important since viviparous mammals evolved. Modern views on fXII are mainly focused on its "negative" effects: (1) thrombus formation and thrombosis development in a wide variety of animal models, and (2) activation of blood clotting during contact with foreign surfaces which complicates cardiac and vascular surgery and interferes with coagulation assays. We have emphasized a demand on a highly active and selective inhibitor of the contact activation and considered the currently known methods to block fXIIa. Here we review the endogenous plasma inhibitors and inhibitors from natural sources, as well as engineered compounds — antibodies, aptamers, phage display-selected peptides. Also, the ASO method of thrombosis prevention is mentioned. Low-molecular weight compounds are not covered with this review because of the lack of compounds with a reasonable selectivity. Further, we compare these inhibitors and describe their shortcomings. From a variety of methods of fXIIa inhibition, we choose the most perspective ones, including RNA-aptamer which selectively blocks both fXII auto-activation and binding of fXI to fXIIa (promoting activation of the intrinsic pathway) via its negative surface-binding domain. Finally, we make demand on the search of new inhibitors and outline the methods for their discovery, placing emphasis on a selection of the ligands which selectively block fXI- and/or anionic surfaces-binding sites of the regulatory domains of fXII.

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