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ARTICLES

The Role of Transglutaminases in the Regulation of Phosphatidylserine-Positive Platelet Formation

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Abstract—Platelets, specialized blood cells, are the most important members of the hemostatic system. Upon activation they divide into two subpopulations, differing greatly in their properties. Platelets from one of these subpopulations express phosphatidylserine (PS) on the outer layer of their membrane and retain secreted alpha-granule proteins on their surface. We inquired if transglutaminases participate in regulation of the formation of platelet subpopulations. Synthetic peptides F11KA and T26 inhibiting factor XIIIa and tissue transglutaminase, respectively, dansylcadaverine, and competitive inhibitors/substrates (serotonin, cystamine, and GTP) reduced the amount of PS-positive platelets. However, inhibitory antibody against tissue transglutaminase did not alter the number of PS-positive platelets, and influence of pan-transglutaminase inhibitor T101 was moderate (20% for the activation by thrombin with collagen-related peptide (CRP)) and statistically insignificant ($p = 0.08$). The addition of transglutaminases caused a statistically significant increase in the number of PS-positive platelets (by 27% and 14% for tissue transglutaminase and factor XIIIa, respectively) upon activation with CRP but not with thrombin. Taking into account a well-known non-specificity of most inhibitors used in this study and their high effective concentrations, we suggest that transglutaminases may significantly influence the formation of procoagulant platelets in some conditions (upon platelet stimulation via collagen receptor GPVI). However, our data do not support the idea that transglutaminases can play a major and universal role in this phenomenon.

Keywords: platelets, platelet subpopulations, tissue transglutaminase, factor XIIIa, phosphatidylserine, flow cytometry, aggregometry

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INTRODUCTION

Hemostasis is a defense system, which ceases bleeding upon a blood vessel rupture. Platelets are the most important component of hemostasis. Investigations of the last two decades have shown that upon strong activation platelets divide into several subpopulations, which differ in their properties dramatically [1, 2]. Platelets from one of these subpopulations express high level of phosphatidylserine (PS) on their surface, where they also retain several alpha-granule proteins, e.g., fibrinogen, thrombospondin, and von Willebrand factor. These proteins are assumed to be substrates for cellular transglutaminases [3] – γ-glutamyltransferases [EC 2.3.2.13] catalyzing the formation of ε-(γ-glutamyl)lysine cross-links between proteins [4].

Platelets contain two types of transglutaminases: tissue transglutaminase [5] and factor XIIIa [6]. Data regarding their influence on the number of PS-positive platelets forming upon activation are contradictory. Dale et al. [3] showed that antibodies, which inhibit factor XIIIa and block its activation by thrombin, suppress the formation of the platelet subpopulation whose surface is coated with alpha-granular proteins. Our previous study of the mechanism driving the formation of this protein coat confirmed that transglutaminases can indeed promote a formation of the coat, although it seemed that in most cases another mechanism is employed [7]. However, in these studies a protein coat was examined rather than the PS exposure. Moreover, Kulkarni and Jackson [8] showed that factor XIIIa can mediate a transition of platelets from

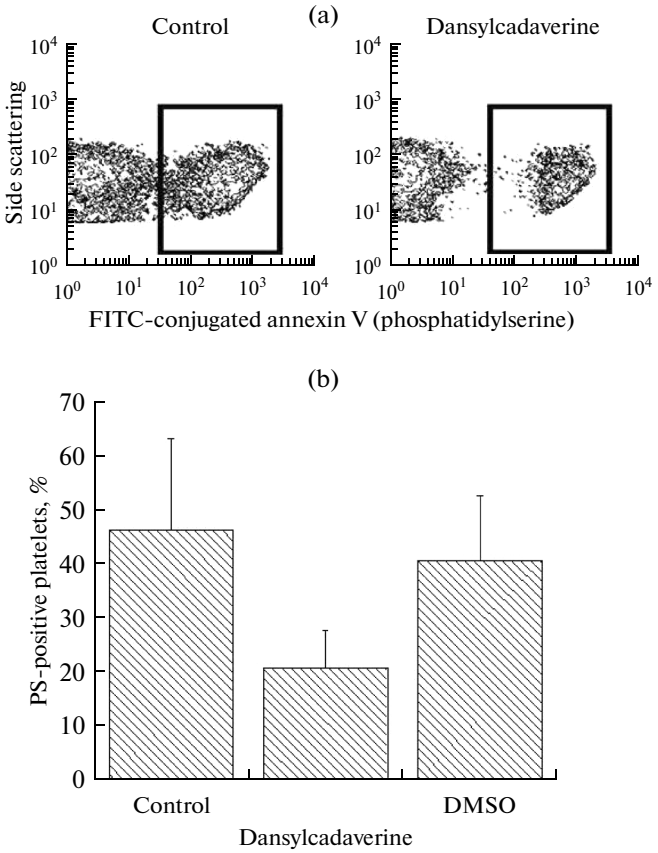

Fig. 1. The effect of transglutaminase inhibitor dansylcadaverine on the formation of PS-positive platelets. Platelets (2×10^4/μL) were activated by 10 nM thrombin with 10 ng/mL convulxin in the presence of 200 μM dansylcadaverine. Control: samples without inhibitor, in the presence or absence of the solvent dimethyl sulfoxide (DMSO). (a) Typical dot plot for the platelets activated in the presence or absence of dansylcadaverine. The boxed area indicates the PS-positive platelet subpopulation. (b) Histogram of the percentage of PS-positive platelets. Mean values and standard error of the mean ($n = 3$) are shown.

proadhesive to procoagulant type (although the attention was mostly payed to platelet morphology). In contrast, factor XIIIa-deficient mice suffering from hemorrhagic diathesis (including high frequency of spontaneous bleeding and intrauterine death of fetus), similar to the human manifestations of factor XIIIa deficiency, exhibited normal formation of PS-positive platelets and normal expression of P-selectin (membrane protein of alpha-granules) [9].

Here, we aimed to study the role of transglutaminases in regulation of PS-positive platelet formation upon platelet activation.

MATERIALS AND METHODS

Materials. The following reagents were used: human thrombin, coagulation factors IXa, X, and XIIIa (Haematologic Technologies, USA; Roche, France); convulxin (Pentapharm, Switzerland); prostaglandin E1 (MP Biochemicals, USA); fluorescein isothiocyanate (FITC)-conjugated annexin V (Molecular Probes, USA); PPACK (Calbiochem, USA); human factor VIII (Hemophil M, Russia); chromogenic substrate S2765 (Chromogenix, Italy); inhibitor T101 (ZEDIRA, Germany); mouse anti-human transglutaminase II IgG1 antibody and isotype control IgG1 (Thermo Scientific, USA), which were preliminarily dialyzed to remove sodium azide; inhibiting peptides T26 (HQSYVDPWMLDH) and F11KA (DQMMLPWPAVAL) against human tissue transglutaminase and human factor XIIIa, respectively [10], which were synthesized in the Laboratory for Protein Engineering (Orekhovich Institute of Biomedical Chemistry, Moscow, Russia). Collagen-related peptide (CRP) was a generous gift from Prof. R.W. Farndale (University of Cambridge, UK). Other reagents were from Sigma–Aldrich, USA.

Platelet isolation. Investigation was performed in accordance with the Helsinki Declaration and under the approval of the Ethical Committees of the Center for Theoretical Problems of Physicochemical Pharmacology and National Research Center for Hematology. Written informed consent was obtained from all donors. Platelets were isolated from blood of healthy volunteers, collected into 3.8% citrate buffer (111 mM sodium citrate, pH 5.5, blood : citrate volume ratio was 9 : 1); also prostaglandin E1 (1 μM) and apyrase (0.1 U/mL) were added to the blood to prevent platelet activation. Blood was centrifuged at room temperature at 100 *g* for 8 min; the obtained platelet-rich plasma was supplemented with 3.8% citrate buffer (plasma : citrate volume ratio 3 : 1). Platelets were concentrated by centrifugation at room temperature at 400 *g* for 5 min, resuspended in 300 μL of buffer A (150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20 mM HEPES, 5 mM glucose, 0.5% bovine serum albumin, pH 7.4) and subjected to gel filtration to remove plasma proteins on a chromatography column (diameter × height = 1 cm × 6 cm) packed with Sepharose CL-2B and equilibrated with buffer A.

Flow cytometry. Platelets at indicated concentrations were stimulated by incubation with agonists and fluorescently labeled antibodies in buffer A containing 2.5 mM $CaCl_2$ for 15 min, diluted either 20- or 10-fold with buffer A containing 2.5 mM $CaCl_2$ and analyzed in a flow cytometer FACSCalibur (BD Biosciences, USA) or Accuri C6 (Accuri Cytometers, USA), respectively. The acquired data were processed using a WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, USA) and CFlow program (Accuri Cytometers, USA). The compensation procedure (correction of the fluorescence spectra overlap) was performed in all cases of two- or three-color analysis.

Aggregation. Platelets at a concentration of 10^5/μL in buffer A containing 2.5 mM $CaCl_2$ were preincubated with either 200 μM dansylcadaverine or vehicle

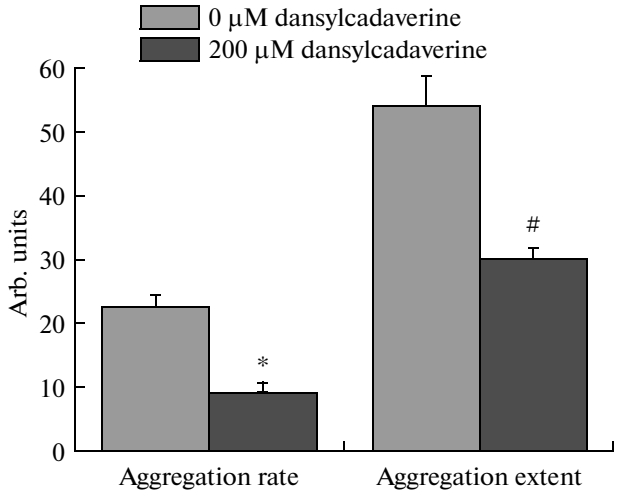

Fig. 2. The influence of dansylcadaverine on platelet aggregation. Histograms of aggregation parameters (rate and extent) for platelets activated with 10 nM thrombin with 10 ng/mL convulxin in the absence or presence of 200 μM dansylcadaverine are presented. Mean values and standard error of the mean ($n = 3$) are shown. *, # The value significantly differs from the respective control at $p < 0.05$.

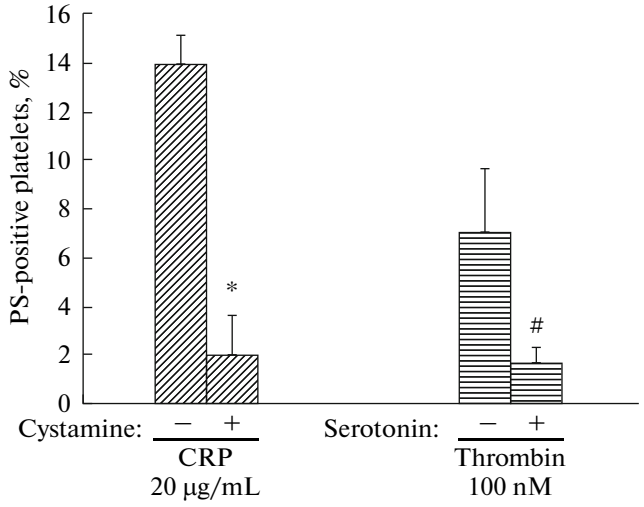

Fig. 3. The effect of competitive transglutaminase inhibitors on the formation of PS-positive platelets. Platelets ($5 \times 10^4/\mu L$) were activated for 15 min at 37°C with 100 nM thrombin or 20 μg/mL CRP with the addition of 10 mM cystamine or 1 mM serotonin. Mean values of PS-positive platelet number and standard deviation ($n = 3$) are shown. *, # The value significantly differs from the respective control at $p < 0.05$.

(0.125% DMSO) for 3 min at 37°C. Then they were mixed with 10 nM thrombin and 10 ng/mL convulxin, and platelet aggregation was monitored with aggregometer Chrono-Log 490 (Chrono-log; Havertown, USA) for 20 min while stirring (800 rpm) at 37°C. Two parameters were estimated from the obtained time-course curves of the light transmission: the extent (maximal increase of light transmission during the experiment) and the rate of aggregation (maximal slope of aggregatory curve at approx. 30% of the linear range).

Measurement of the platelet procoagulant activity. The intrinsic tenase complex was assembled on the platelet surface. Stimulated platelets ($8 \times 10^3/\mu L$) in buffer A containing 2.5 mM $CaCl_2$ were mixed with 27.5 nM factor VIIIa (factor VIII was preliminary activated for 1 min by thrombin, which was then blocked with 2 μM PPACK), 10 nM factor IXa, and 400 nM factor X. Tenase complex activated factor X for 4 min at 37°C. Then 100 mM EDTA was added to the suspension to stop the reaction of factor X activation followed by the addition of 0.4 mM S2765, a chromogenic substrate for factor Xa. Initial rate of the substrate hydrolysis was evaluated by changes in optical density at 405 nm monitored at 37°C for 30 min using the plate reader Thermomax (Molecular Devices, USA).

Prior to the assembly of intrinsic tenase complex, platelets were stimulated with 100 ng/mL convulxin, and 500 nM tissue transglutaminase was added to the suspension either immediately or 9 min after the start of activation (full time of activation was 15 min). Control samples contained platelets, which were either non-stimulated or stimulated with 100 ng/mL convulxin alone.

Statistical analysis was performed using paired Student's *t*-test in OriginPro 7.5 (Microcal Software, USA).

RESULTS AND DISCUSSION

We studied the role of transglutaminases in regulation of the amount of PS-positive platelets using a full spectrum of available pharmacological compounds with various anti-transglutaminase activities.

First, we used dansylcadaverine, a competitive amino donor for transglutaminases, which inhibits both tissue transglutaminase and factor XIIIa. This inhibitor was used in previous studies [3, 8, 11]. The addition of 200 μM dansylcadaverine caused a twofold reduction in the amount of PS-positive platelets as compared to the control (Fig. 1). These data are consistent with the observations of Dale et al., who reported that the addition of 200 μM dansylcadaverine led to a 5-fold decrease in the total amount of alpha-granular proteins on the membrane of the PS-positive platelets [3, 11]. Additional experiments showed that the solvent alone did not exert any effect on platelets. We showed previously that a reduction in procoagulant platelet percentage in a suspension improves the aggregation, since PS-positive platelets do aggregate but only with PS-negative ones [12]. Therefore, by means of optical aggregometry we tested the influence of dansylcadaverine on the platelet aggregation. Paradoxically, the addition of 200 μM of this inhibitor that decreases the number of PS-positive platelets down-

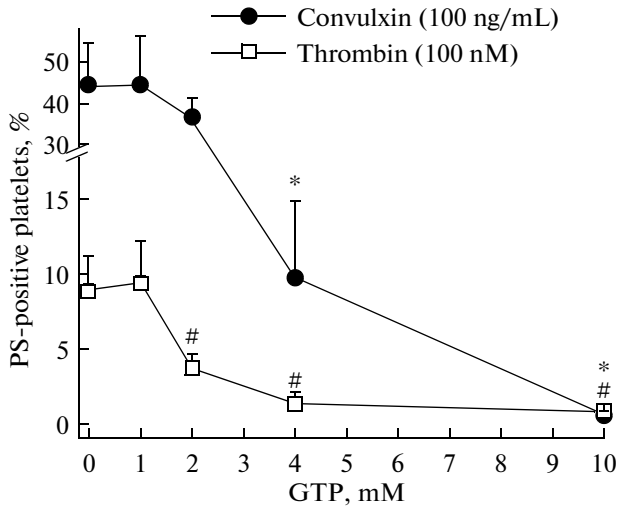

Fig. 4. The influence of GTP on the formation of PS-positive platelets. Platelets ($5 \times 10^4/\mu L$) were activated with convulxin (100 ng/mL; *black dots*) or thrombin (100 nM; *unfilled squares*). Mean values and standard error of the mean ($n = 3$) are shown. *, #The value significantly differs from the respective value at 0 mM GTP, $p < 0.05$.

regulated the aggregation (Fig. 2); the aggregation rate was reduced by 59.9% ($p < 0.05$) and the aggregation extent, by 44.1% ($p < 0.05$). These data challenge the results obtained with dansylcadaverine, since they show that this substance not only influences the number the PS-positive platelets, but also changes their properties dramatically, suggesting a considerable non-specificity of dansylcadaverine that becomes apparent at high concentrations.

Furthermore, we used such competitive transglutaminase inhibitors as cystamine and serotonin. They are protein substrates for transglutaminases that either inhibit the transamidation reaction (as cystamine does) [13] or compete for the ε-lysine cross-link (as serotonin), thus leading to the inhibition of physiological transglutaminase activity [14]. Addition of either cystamine or serotonin to the platelet suspension caused a statistically significant ($p < 0.05$) reduction in the amount of PS-positive platelets upon stimulation with CRP or thrombin (Fig. 3).

GTP binding with tissue transglutaminase induces its conformational changes and inhibition of its enzymatic activity [15]. Addition of GTP to the platelet suspension also caused a dose-dependent reduction in the number of PS-positive platelets (Fig. 4).

At the next step we used synthetic peptides T26 and F11KA, which were reported to selectively inhibit tissue transglutaminase and factor XIIIa, respectively [10]. We used these peptides to reveal the role of individual transglutaminase in regulation of the PS-positive platelet number upon platelet activation. In the case of T26 peptide (inhibiting tissue transglutaminase), the

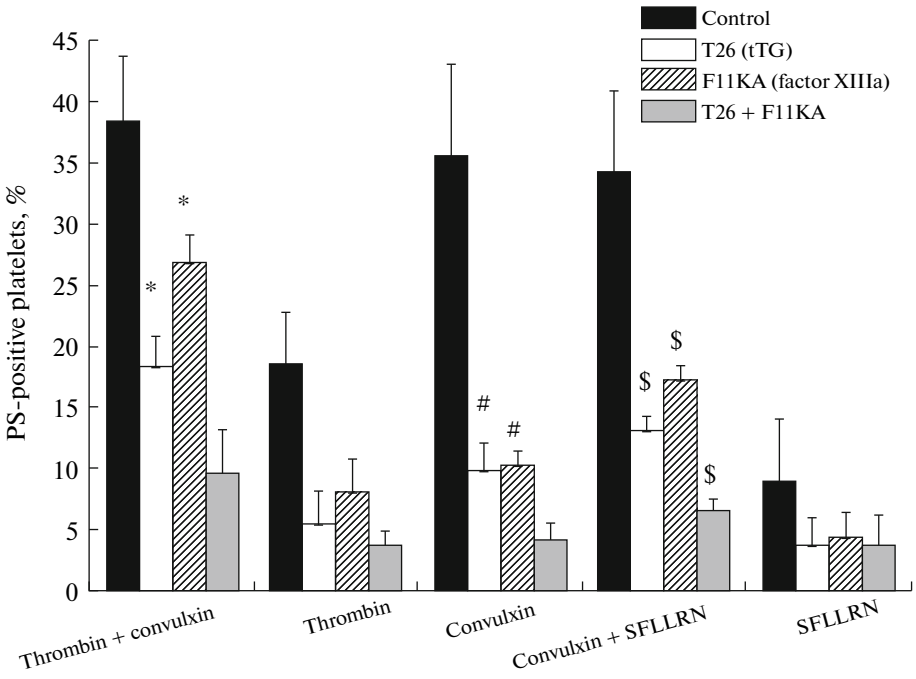

Fig. 5. The effect of inhibition of tissue transglutaminase (tTG) and/or factor XIIIa during PS-positive platelet formation. Platelets ($10^5/\mu L$) were preincubated for 30 min with 1 mM T26 (tTG inhibitor) and/or F11KA (factor XIIIa inhibitor) and then they were activated by 10 nM thrombin with 1 ng/mL convulxin, 100 nM thrombin, 10 ng/mL convulxin, 1 ng/mL convulxin with 150 μM SFLLRN (thrombin receptor PAR-1 agonist), or 150 μM SFLLRN. Mean values and standard error of the mean ($n = 3$) are shown. *, #, $ The value significantly differs ($p < 0.05$) from the corresponding control without inhibiting peptides.

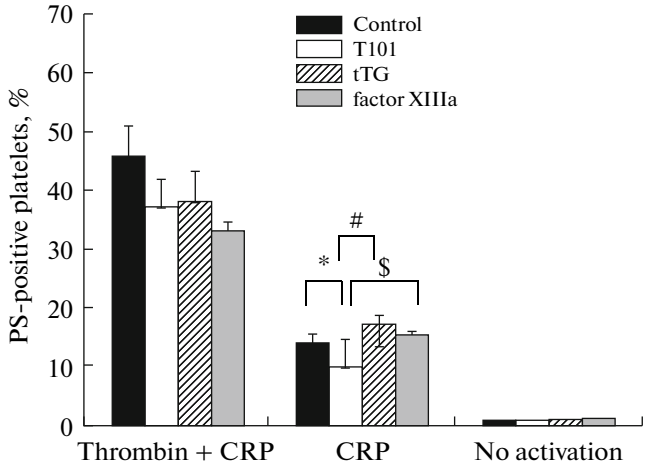

Fig. 6. The role of transglutaminases in the regulation of the number of PS-positive platelets. Platelets (10^5/μL) were activated for 15 min by 100 nM thrombin with 10 ng/mL CRP or 20 ng/mL CRP in the presence of 200 μM pan-transglutaminase inhibitor T101, 500 nM tissue transglutaminase (tTG), or 500 nM factor XIIIa. Mean values and standard error of the mean ($n = 4$) are shown. *The difference between the values in the presence of inhibitor T101 and in its absence is statistically significant at $p < 0.05$. #The difference between the values in the presence of tTG and inhibitor T101 is statistically significant at $p < 0.05$. $The difference between the values in the presence of factor XIIIa and inhibitor T101 is statistically significant at $p < 0.05$.

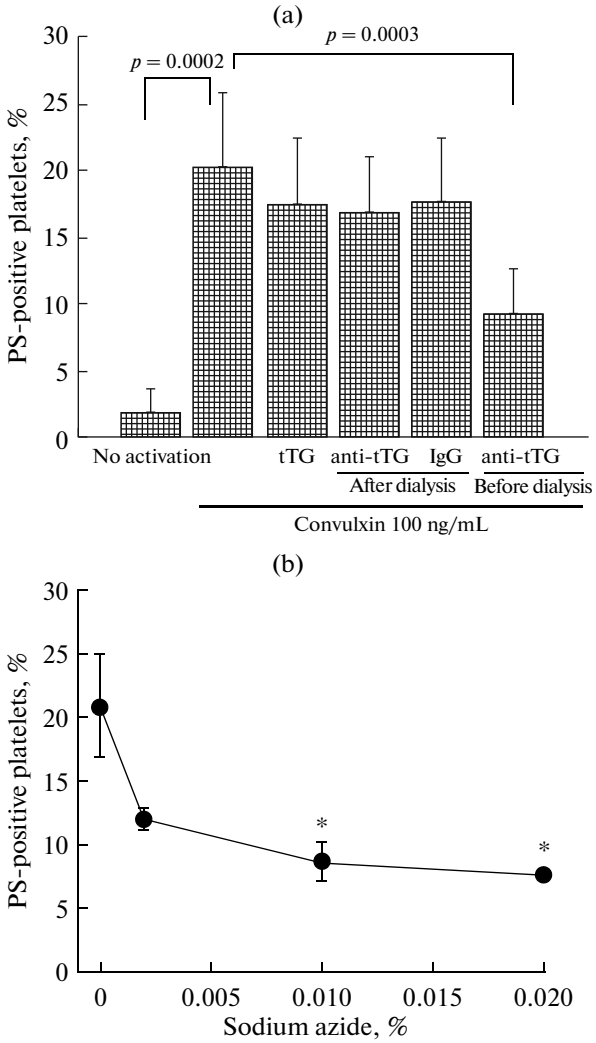

Fig. 7. The effect of the antibody against tissue transglutaminase (anti-tTG) on the formation of PS-positive platelets. (a) Platelets (5×10^4/μL) were activated for 15 min at 37°C by 100 ng/mL convulxin in the presence of either 500 nM tTG, 150 nM anti-tTG before the dialysis (buffer contained 0.02% sodium azide), 150 nM anti-tTG after the dialysis (buffer contained no sodium azide), or 150 nM control IgG after the dialysis. Mean values of PS-positive platelet number and standard deviation ($n = 3$–6) and p-values for the comparison of corresponding groups are shown. (b) The effect of sodium azide on the formation of PS-positive platelets. Platelets (5×10^4/μL) were activated for 15 min at 37°C by 100 nM thrombin with the addition of sodium azide at indicated concentrations (%). Non-dialyzed 150 nM anti-tTG solution contained 0.02% sodium azide. Mean values ± standard deviation ($n = 3$) are shown. *$p < 0.05$.

level of PS-positive was platelets was reduced 2–4-fold at all types of stimulation ($p < 0.05$ upon stimulation with convulxin and thrombin, convulxin and SFLLRN, or convulxin alone) as compared to the control value. Similarly, F11KA (inhibiting FXIIIa) reduced the number of PS-positive platelets 1.5–3.5-fold ($p < 0.05$ upon stimulation with convulxin and thrombin, convulxin and SFLLRN, or convulxin alone). Inhibition of both transglutaminases reduced the number of PS-positive platelets 2.5–8.5-fold at different types of activation; nevertheless, this platelet subpopulation did not disappear completely (Fig. 5). These results indicate that both factor XIIIa and tissue transglutaminase participate in the formation of procoagulant platelet subpopulation upon platelet activation. However, at millimolar effective concentrations the peptides should be suspected of non-specificity, and negative controls for them do not exist.

In order to strictly investigate the effect of transglutaminase inhibition or the addition of exogenous transglutaminases on the formation of the PS-positive platelet subpopulation, we added pan-transglutaminase inhibitor T101 [16], FXIIIa, or tissue transglutaminase to the platelets activated with CRP plus thrombin or CRP alone. Irreversible pan-transglutaminase inhibitor T101 only slightly reduced the number of PS-positive platelets, and a statistically significant reduction by 30% ($p < 0.05$) was observed only upon activation with CRP (Fig. 6). Addition of the exogenous transglutaminases, either tissue transglutaminase or factor XIIIa, led to a statistically significant increase in the number of PS-positive platelets, but again, only upon stimulation with CRP, by 27 and 14% ($p < 0.05$), respectively.

Along with the synthetic peptides, whose specificity could be judged only from the data of [10], we used a

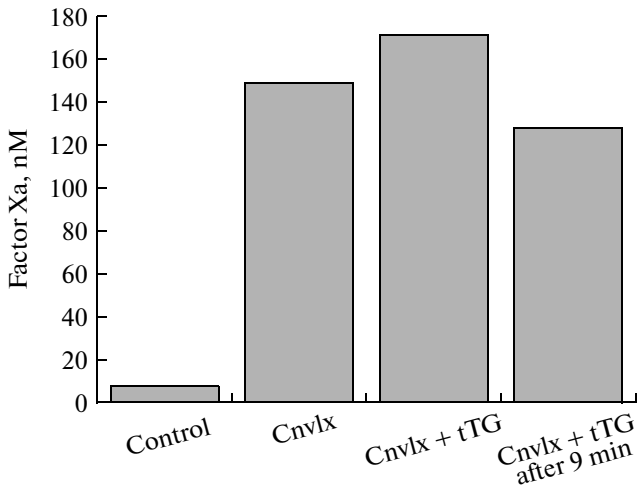

Fig. 8. The influence of tissue transglutaminase (tTG) on the activity of intrinsic tenase complex assembled on the platelet surface. Platelets were either non-stimulated or stimulated by 100 ng/mL convulxin (Cnvlx) in the absence or presence of 500 nM tTG added to the suspension immediately or 9 min after the start of platelet activation. The result of a typical experiment is shown ($n = 2$).

well defined and highly specific antibody against tissue transglutaminase to reveal its role. The antibody was preliminarily dialyzed to change the buffer, which contained bacteriostatic agent sodium azide. In contrast to the inhibiting peptide T26, dialyzed antibody had no effect on PS-positive platelets (Fig. 7a). At the same time, non-dialyzed antibody inhibited the formation of procoagulant platelet subpopulation (Fig. 7a). Such an effect is apparently caused by the presence of sodium azide in the initial buffer, since this bacteriostatic preservative reduces PS-positive platelet number in a dose-dependent manner (Fig. 7b).

Finally, to study the influence of transglutaminases on the functional activity of procoagulant platelets (and not only on their number), we investigated the impact of tissue transglutaminase on the activity of the intrinsic tenase complex assembled on the surface of these platelets. Addition of tissue transglutaminase did not lead to an increase in factor Xa production, indicating that tissue transglutaminase has no effect on the platelet procoagulant activity (Fig. 8).

Taking into account a well-known non-specificity of most inhibitors used in this study and their high effective concentrations, we sugget that transglutaminases may significantly influence the formation of procoagulant platelets in some conditions (upon platelet stimulation via collagen receptor GPVI). However, our data do not support the idea that transglutaminases can play a major and universal role in this phenomenon.

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