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Transglutaminase 2 contributes to the blood clotting cascade

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Abstract

The cross linking of fibrin and associated proteins via Nε-(γ-glutamyl)lysine bonds in clot formation is a well-established component of the haemostatic mechanism, providing clot rigidity and reducing the rate of fibrinolysis. The latter effect occurrs predominately through the incorporation of fibrinolytic inhibitors. To date this function has been ascribed exclusively to Factor XIIIa also known as plasma transglutaminase (TGs), one of eight identified in mammals. In this report we demonstrate that tissue transglutaminase (TG2), a ubiquitous enzyme abuntantly present in erythrocytes, also contributed to clot stabilisation. We observed clear differences between whole thrombi isolated from TG2-deficient and wild type mice. Similarly, human thrombi were more stable in the presence of TG2, than when TG2 activity is specifically inhibited, revealing a previous unrecognized role for this enzyme in haemostasis. We therefore propose TG2 as an additional factor involved in blood coagulation.

Introduction

The crosslinking of fibrin is an essential part of the haemostatic mechanism [1–4].To date fibrin stabilisation has been solely ascribed to Factor XIIIa, a plasma transglutaminase (EC 2.3.2.13; TG) also found in platelets and monocytes [5].TGs are a family of enzymes which crosslink proteins via N^{ε}(γ-glutamyl)lysine isopeptide bonds, leading to the formation of high molecular weight aggregates with increased rigidity and enhanced stability to proteolysis and chemical degradation [5–7].

The epidermis shows the presence of several TG, such as TG1[8] or TG5 [9], that participate in the formation of the cornified envelope[10] and therefore to tissue homeostasis [11, 12]. Deregulation of this process results in specific genetic diseases such as lamellar ichthyosis [8]. Conversely, TG2 is the most ubiquitously expressed and functionally versatile isoform, displaying a unique combination of enzymatic and non-enzymatic activities, reviewed in [13–15]. Indeed, TG2 can be activated by serotonin[16] in vascular smooth muscle cells [17], facilitating protein kinase A-induced phosphorylation of retinoblastoma protein[18] and can contribute to pulmonary inflammation and fibrosis in bleomycin-treated mice [19, 20]. Additional recent evidences, thanks to its crosslinking properties, has also implicated TG2 in cell death modalities [21]as well as in Parkinson [22].

Abnormal blood clotting has been found in humans carrying genetic defects of Factor XIIIa [23]. However this genetic defect is minor when compared to the phenotype of patients defective for Factor VIII or IX. A possibile hypthesis indicates that there could be that another TG that partially compensates the defects of Factor XIIIa. Indeed, TG2, is implicated in wound healing[24] and it is highly abundant in erythrocytes and endothelial cells [5, 25], therefore it is present in clotted blood. Unlike Factor XIIIa, TG2 does not require proteolytic activation, it is found both in the intracellular and extracellular compartments, and under normal physiological conditions its Ca^{2+} activated transamidating activity is kept silent inside the cell by its binding of GTP/GDP[26] or NO [27]. TG2 deficient mice[28] show minor phenotypes [29–32], one of which is slowed wound healing. In this report we provide, evidence for the involvement of TG2 in the blood clotting cascade.

Results

Model thrombi[33] from TG2 null mice are more susceptible to hemolysis than thrombi prepared from wild type mice (Fig. 1A). The rate of hemolysis was 2–3 fold greater ($p < 0.001$) than wild type animals and approximately 50% of the rate of hemolysis when the TG specific inhibitor R283 was included in the clot mixture. Consistent with this change in hemolysis was the levels of N^{ε} (γ-glutamyl) lysine crosslink found in proteolytic digests of thrombi which was approximately 50% less ($p = 0.05$, 95%) in the TG2 KO mice, see Fig. 1B.

The presence of TG2 in the clot was confirmed in Fig. 2A-B by the staining of human model thombi using a specific TG2 antibody. PCR analysis of leucocytes did not reveal the presence of TG2 mRNA and platelets contain very little TG2, suggesting that the erythrocyte is the major provider of TG2 to clot stabilisation.

To further demonstrate the involvement of TG2 in thrombus stabilisation in humans, thrombi were prepared separately from the blood of six normal human donors, incubated with the non hydrolysable GTP analogue GTPγS. At 1mM this nucleotide inhibits the Ca^{2+}-activation of TG2 but not that of Factor XIIIa, at the levels of Ca^{2+} used in the formation of the model thrombi [26]. Inclusion of GTPγS in the thrombi resulted in a 1.3 fold faster rate of lysis than control thrombi, as shown in Fig. 2C. Inhibition of all TG mediated crosslinking by the specific active site directed inhibitor R283 resulted in even faster lysis (1.7 fold, $p < 0.005$). This is consistent with the crosslink levels and lysis data obtained from the mouse thrombi, and indicates the TG2 contribution to clot stabilisation to be comparable to that of Factor XIIIa.

Having provided evidences in genetically modified mice, we then explored the presence of TG2 in 10 trombi collected during mechanical thrombectomy from patients with acute ischemic stroke. Table 1 shows the clinical details of the patients. Both immunofluorescence and immunohistochemical analyses confirmed the presence of TG2 in all human thrombi analyzed (Table 1). Specifically, immunofluorescence revealed TG2 localization within extracellular fibrin-rich regions of the clot (Fig. 3A-F). Immunohistochemistry further allowed the assessment of TG2 expression within the morphological context of the thrombus (Fig. 4A–D).

Table 1
Clinical and immunohistochemical data

Case	Age	Gender	Thrombus site	Extracellular positivity (Score 0–3)	Blood cell positivity (Score 0–3)
1	82	M	anterior cerebral circulation	**3**	2
2	72	M	anterior cerebral circulation	**3**	1
3	57	M	anterior cerebral circulation	1	3
4	86	F	anterior cerebral circulation	**3**	1
5	93	F	anterior cerebral circulation	0	3
6	47	M	anterior cerebral circulation	1	0
7	75	M	anterior cerebral circulation	2	1
8	82	F	anterior cerebral circulation	**3**	2
9	85	F	anterior cerebral circulation	1	2
10	74	F	anterior cerebral circulation	**3**	1

TG2 staining showed marked heterogeneity among samples. All thrombi exhibited TG2 positivity in the extracellular compartment (fibrin-rich regions) and/or within blood cells. With regard to the extracellular compartment (Fig. 4A,C), only one thrombus, obtained from a patient older than 90 years, lacked detectable TG2 staining, whereas 5 of the 10 thrombi showed extracellular TG2 positivity in more than 20% of the fibrin-rich area.

Cellular TG2 expression was also highly heterogeneous, although TG2-positive cells were predominantly represented by cells of the monocyte/macrophage lineage (Fig. 3D). Specifically, 2 out of 10 thrombi contained more than 20% TG2-positive cells, 3 out of 10 showed TG2 positivity in 10–20% of cells, and 4 out of 10 displayed positivity in 1–10% of cells. Only one thrombus was negative for cellular TG2 staining. Immunofluorescence and immunohistochemical analysis demonstrate that TG2 is detectable in human ischemic stroke thrombi, although with substantial inter-patient variability in both extracellular and cellular distribution.

These data provide a clinical relevance for TG2 in human clots, as depicted in Fig. 5.

Discussion

The results herein presented, clearly demonstrate that TG2 participates in the crosslinking and stabilisation of the fibrin network in the initial event of forming the haemostatic plug. This could explain the mild phenotype demonstrated by patients deficient in Factor XIIIa: the presence of TG2 in the blood

cascade mechanism would at least partially compensate for this deficiency. The role of TG2 in erythrocytes has been elusive to date, and the enzyme was never found activated in red cells, nor any *in vivo* substrate identified. Moreover it indicates the importance of this step in clot stabilisation and wound healing as a whole, since mammals have evolved not one but two separate enzymes which are capable of clot stabilisation and which are activated by different mechanisms, one by proteolysis by thrombin, the other by Ca^{2+} after its release following red cell lysis in the clot. This phenomenon is reminiscent of the process that occurs during epidermal terminal differentiation, where several transglutaminases contribute to the formation of the cornified envelope, thereby providing protection to the organism [10]. From an evolutionary standpoint, the conservation and diversification of multiple transglutaminase family members indicate their critical contribution to fundamental biological processes. Their distinct but overlapping functions support key protective mechanisms, including hemostasis, barrier integrity, tissue remodeling, and stress adaptation [34–38], highlighting the evolutionary advantage of maintaining a repertoire of specialized transglutaminases to safeguard organismal homeostasis.

Important physiological differences in the crosslinking specificity of these enzymes have come from studies on purified fibrinogen [39]. Once activated by thrombin, Factor XIIIa rapidly crosslinks γ-chains first into γ-γ dimers and then polymers but it acts slowly in the formation of α-polymers and intermolecular crosslinks between α and γ chains. In contrast TG2 acts rapidly in the formation of α-polymers, and the formation of intermolecular crosslinks between α and γ chains. TG2 crosslinking bridges the Aα (408–421) chain segment to γ (392–406) thus binding the flexible αC domain closer to the fibrinogen backbone .These differences in specificity are likely to be of crucial importance to clot rigidity in the initial formation of the haemostatic plug and in its subsequent stability during formation of the granulation tissue during wound healing. Studies indicate that clot rigidity strongly correlates with the formation of α_n-polymers, αpγq – hybrid crosslinks; and γ multimers [40, 41]. In contrast, the initial formation of γ-γ dimers by factor XIIIa contributes very little to clot rigidity. Both TGs are able to crosslink inhibitors of fibrinolysis to fibrinogen and fibrin but with slightly different preferences. Both enzymes crosslink PA1-2 and TAFI to fibrinogen/fibrin but Factor XIIIa is reported to be more efficient in crosslinking α_2-anti plasmin.

As expected no role for TG2 was observed when plasma was clotted in the absence of blood cells as is commonly the case. These white clots lysed at comparable rates whether prepared from TG2 deficient or wild type mice. This is consistent with the present belief that Factor XIIIa is the major fibrin stabilising factor; a view which arose from studies on plasma alone. Suggestive or correlative evidence linking TG2 to cardiovascular events have been reported previously. Indeed, TG2 seems to protect cardiomyocytes against ischemia/reperfusion injury by regulating ATP synthesis [42]. Accordingly, anti-TG2 antibodies have been detected in acute coronary syndrome [43]. Whilst the original interpretation suggested the involvement of TG2 in the platelets aggregates as a response to the lipid plaque, our new data suggests a causal role of TG2, triggered by the release of few lysed erythrocytes, to expand the red clot, hence forming an overt thrombus. These data seems to involve TG2 into the larger field of cardiovascular diseases [44–46].

While our work was in its final stages of refined preparation, two very recent reports hvae described a role for TG2 in thrombus formation [47, 48]. Boateng and collaborator[47] have shown that TG2 is able to drive fibrin β-chain cross-linking. In their hands, fibrinogen is an efficient nsubstrate for TG2, allowing the formation of β-α cross-links, at glance with the cross-links by Factor XIII (that is α-α, β-β). The authors describe these TG2-directed β-chain cross-links in patients with trauma and not in clots from healthy control individuals. Chahdi and collaborators[48] also provide evidence that TG2 contributes to thrombin generation and clot formation, suggesting a role for TG2 in the fibrin structure and spatial distribution within blood clots. Here too, TG2 promotes faster thrombin generation and clot formation. Their interpreation at the molecular level, however, involves mechanical forces exerted by platelets that allow TG2 to be released by erythrocytes which then cross-links fibrinogen [1, 49]. Our results are in full agreement with these independent reports involving TG2 in thromus formation.

TG2 has been already involved in injured and remodelling cardiac tissues, without evoking any clotting activity, where it seems to modulates cellular phenotype, while contributing to matrix cross-linking [50–53]. Similarily, TG2 seems to exhert angiogenic properties by its transamidation function [25]. However the most direct evidence of interaction between the function of TG2 and clotting factor XIII comes from double knockout mice for both TG2 and FXIII-A, showing a synergistic action in cross-linking activities [54]. In keeping, TG2 moderates the expansion of mouse abdominal aortic aneurysms [55], and TG2 limits the extravasation and the resultant myocardial fibrosis associated with factor XIII-A deficiency [56], further evidencing the Factor XIIIa or TG2 interaction. FXIII A subunit (FXIII-A) knockout mice show that platelet aggregation induced by adenosine diphosphate or collagen was normal, while clot retraction (CR) was lost in the platelet-rich plasma (PRP); in contrast, there was no CR impairment in the PRP of TG2-knockout mice compared with that of wild-type mice, indicating that the enzymatic activity of FXIIIa could potentially be synergistic [57].

During thrombosis, activated platelets, leukocytes, and coagulation factors generate a highly pro-oxidant and pro-inflammatory microenvironment characterized by reactive oxygen species (ROS), cytokine release, calcium influx, and fibrin accumulation. These stimuli strongly induce TG2 expression and activation in endothelial cells [58]. TG2 activation contributes to endothelial dysfunction and cell death [58, 59]: under severe ischemic or inflammatory conditions, excessive TG2 activity may favor apoptosis or necrotic-like cell death, exacerbating vascular occlusion and thrombo-inflammatory injury. These observations suggest that TG2 acts as a stress-responsive regulator at the interface between coagulation, inflammation, and endothelial survival during thrombotic disease [60].

By examining a complete model including whole blood, together with some additional very recent work [47, 48], here, we demonstrate a definitive functional role for TG2 in the blood cascade mechanism, Fig. 5, in keeping with the other roles for this enzyme in wound healing. As a consequence, TG2 could be seen as “Factor XIV”, supporting Factor XIII in the blood cascade mechanism.

Material and Methods

Mice

As previously described [28, 61], hemizygous 14E06 Ig KI mice on a C57Bl/6 background were generated by crossing VDJH14E06 and VJκ14E06 single KI mice. TG2-/- mice were generated as described by De Laurenzi & Melino, VDJH14E06 and VJκ14E06 single KI mice were crossed to Tgm2−/− mice [28]. See also protocols in [62, 63]. Mice were bred in the animal house at the Department of Experimental Medicine (University of Rome Tor Vergata). Animals were housed individually in an environmentally controlled room (18–21°C, 40–70% relative humidity) with ad libitum access to water and food and kept under a 12-h dark/12-h light cycle. All animal procedures were approved by the Ethical Commission of the University of Rome “Tor Vergata” and performed according the principles established by the National Institutes of Health and by the Italian Ministry of Health.

Thrombi

Blood samples were collected from normal or knockout mice with their consent into 0.1 vol of 0.13 m trisodium citrate, as described [33, 64]. Plasma that was essentially free of platelets was prepared by centrifugation at 1860 g for 30 min at 4°C. Normal pool plasma, used in clot lysis experiments, was from 3 wild type or 3 knockout mice [33]. For Clot lysis assay, as described [33], pooled plasma in the presence and absence of CPI and of the plasminogen activators tPA (Genentech, San Francisco, CA, USA), uPA (NIBSC) or scuPA (Abbott, Maidenhead, Berks, UK), or plasmin (NIBSC, Potter's Bar, UK) was prepared in a final volume of 200 µL 10 mm Tris pH 7.5, 0.01% Tween 20 (Tris–Tween buffer). Two 80 µL aliquots from each tube were added to microtiter wells, each containing 20 µL CaCl2 to give final concentrations in the wells as follows: 30% plasma, 10.6 mm CaCl2; 180 pm tPA, 1 nm uPA, 8 nm scuPA or 1.25 U mL − 1 plasmin; ± 25 µg mL − 1 CPI. The plate was incubated at 37°C and read continuously at 405 nm [33, 64].

Cross-linking experiments

Cross-linking experiments were performed as previuosly described [65, 66]. See also protocols in [67]. Briefly, enzymatic activity was measured by [^{3}H]putrescine (Amersham Corp., specific activity 26 Ci/mmol) incorporation into succinylated casein, and the amount of TG2 that incorporated 0.45 pmol/min into the casein was used. The cross-linked products were separated on 4–12% polyacrylamide gels and analyzed by autoradiography [68, 69]. The Nε-(γ-glutamyl)lysine crosslink was analysed as detailed in the legend to Fig. 1: Cross-links in blood clots. Whole blood clots were formed with blood from TG2$^{-/-}$ and WT (TG2$^{+/+}$) mice. Clots were digested and analysed by HPLC. For analysis of the Nε-(γ-glutamyl)lysine isodipeptide bonds, clots were first washed with PBS containing 5mM EDTA and then with PBS alone. The clots were then lyophilised resuspended in 0.1M ammonium hydrogen carbonate pH 8 buffer and then proteolytically digested) and analysed using cation exchange chromatography as described by Griffin and Wilson, [69].

Human cohort

Ten thrombi were collected during mechanical thrombectomy from patients with acute ischemic stroke caused by large vessel occlusion of the intracranial anterior circulation, see Table 1. As previously described [64], thrombi were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial 4-µm-thick sections were prepared for hematoxylin and eosin staining, immunofluorescence, and immunohistochemical analyses.

For immunofluorescence analysis, tissue deparaffinized sections were rehydrataded in graded alchool and immersed for 1 h in 70% ethanol supplemented with 0,25% NH_3 to remove tissue autofluorescence [70]. Slides rehydration was resumed by immersion in 50% ethanol for 10 min, after wich the sections were washed with distilled H_2O and underwent heat-induced antigen retrieval in EDTA-citrate buffer (pH 6.8) using a pressure cooker at 120°C for 15 min. Sections were then blocked for 1 h at room temperature with a 5% goat serum and than incubated for 1 h with mouse monoclonal anti-TG2 antibody (1:500; clone CUB 7402; Invitrogen). Immunoreactivity was detected using Alexa fluor 647 as anti-mouse secondary antibody (thermo fisher) and DAPI (Sigma Aldrich) was used to counterstain cells nuclei. Sections fluorescence was detected and acquired by using confocal microscope Leica Stellaris 5 and images analysed with LASX Leica software.

Immunohistochemical staining was performed using the automated Leica Bond III staining platform (Leica Biosystems). The same primary anti-TG2 antibody used for immunofluorescence (1:1000; clone CUB 7402; Invitrogen) was applied under identical conditions. Immunoreactivity was visualized using a horseradish peroxidase (HRP)-based detection system with 3,3'-diaminobenzidine (DAB) as the chromogen. TG2 immunoreactivity was assessed separately in the extracellular fibrin-rich component and in the cellular component of each thrombus using a semi-quantitative pathological scoring system. For the extracellular compartment, staining was scored as follows: score 0, no TG2-positive fibrin-rich areas; score 1, TG2 positivity involving 1–10% of the fibrin-rich area; score 2, TG2 positivity involving 11–20% of the fibrin-rich area; and score 3, TG2 positivity involving > 20% of the fibrin-rich area. For the cellular compartment, staining was scored as follows: score 0, absence of TG2-positive cells; score 1, TG2-positive cells accounting for 1–10% of the cellular population; score 2, TG2-positive cells accounting for 11–20% of the cellular population; and score 3, TG2-positive cells accounting for > 20% of the cellular population.

Declarations

Compliance with Ethical Norms

All the procedures carried out in the research with participation of humans were in compliance with the ethical standards of the institutional and/or national ethics committee and with the Helsinki Declaration of 1964 and its subsequent changes or with comparable ethics standards. Informed voluntary consent

was obtained from every participant of the study. Animal work was according to both UK Home Office and Italian Ministry of Health regulation, respecting ethics and safety for the mice. All authorizations were obtained for specific strains and detailed experiments (Prot n°219.24). Mice are now deposited at the European consortium EMMA.

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Authors’ contributions

MG and GM conceived the project, GM, MG, MP and EC wrote the manuscript; MG, EC prepared Figs. 1 and 2; MM prepared Fig. 3; AM, MS prepared Fig. 4; GM prepared Fig. 5. All of the Authors have approved this submitted version.

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Conflict of interest.

The authors declare no other conflict of interest

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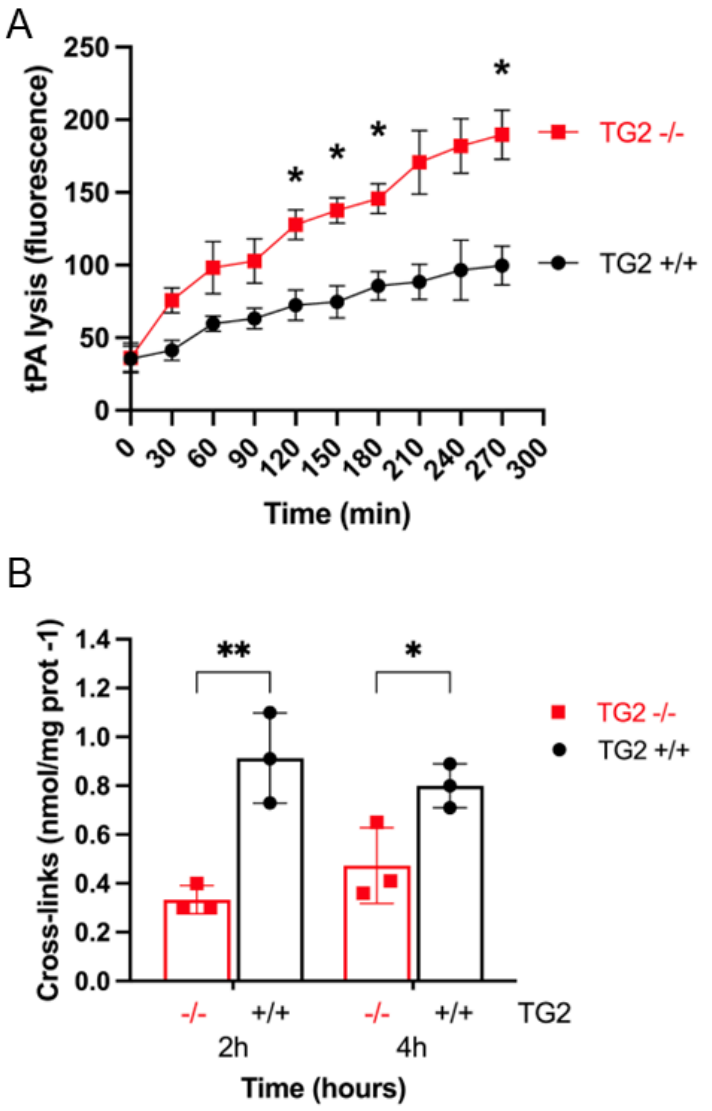
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Figures



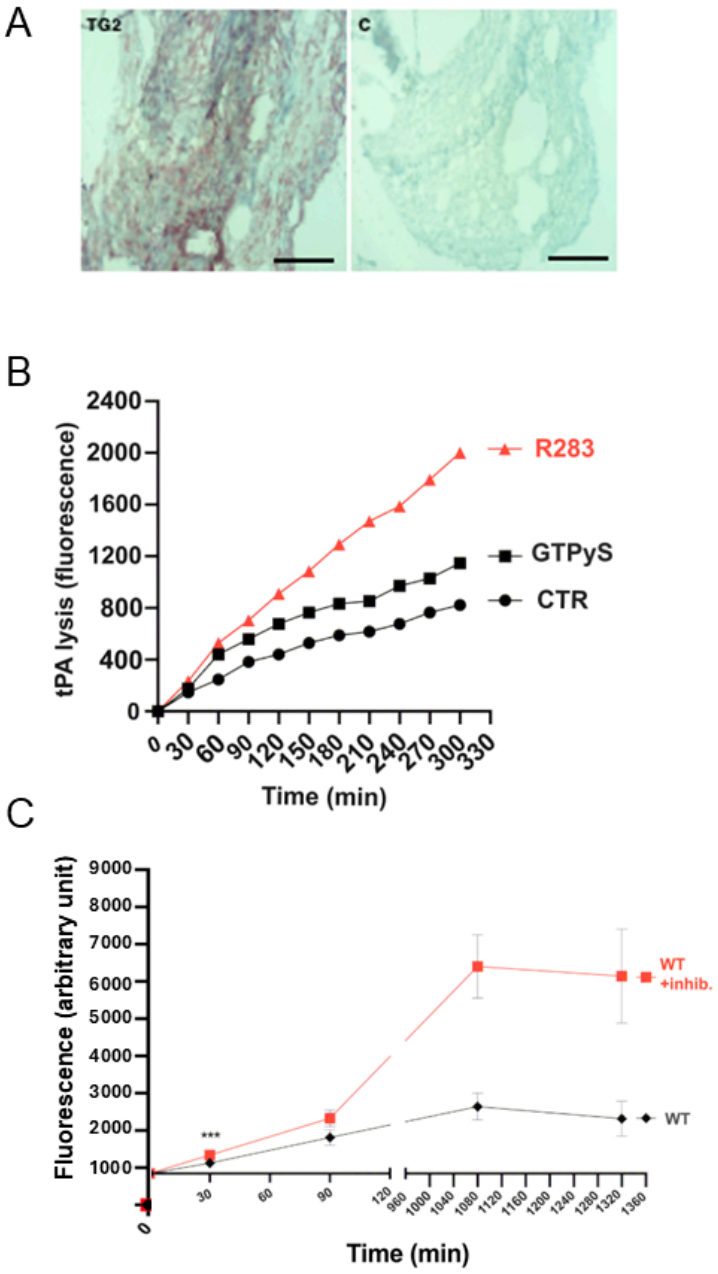
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Figure 1

Figure 1

TG2 activity in mice.

(a) The role of TG2 in thrombus stability. Thrombi were formed with blood from three WT ($TG2^{+/+}$) and three $TG2^{-/-}$ mice and lysed by tPA (250 ng/ml); the data shown are typical of three separate experiments each with n.3 WT ($TG2^{+/+}$) and n.3 $TG2^{-/-}$ mice, with a significant difference in rate of lysis at

the indicated time points (*p<0.05). Thrombi were bathed in 10 mM Tris pH 7.5, 0.01 % Tween 20 containing 1 mg/ml tPA. Thrombi from mouse blood (drawn by cardiac puncture into 0.1 vol of 0.13 M trisodium citrate) were lysed by addition of 250 ng/ml tPA. **(b)** Cross-links in blood clots. Whole blood clots were formed with blood from $TG2^{-/-}$ and WT ($TG2^{+/+}$) mice. Clots were digested and analysed by HPLC. For analysis of the e(g-glutamyl)lysine isodipeptide clots were first washed with PBS containing 5mM EDTA and then with PBS alone. The bars represent analyses from three WT ($TG2^{+/+}$) and three $TG2^{-/-}$ mice *p < 0.05, **p < 0.001.



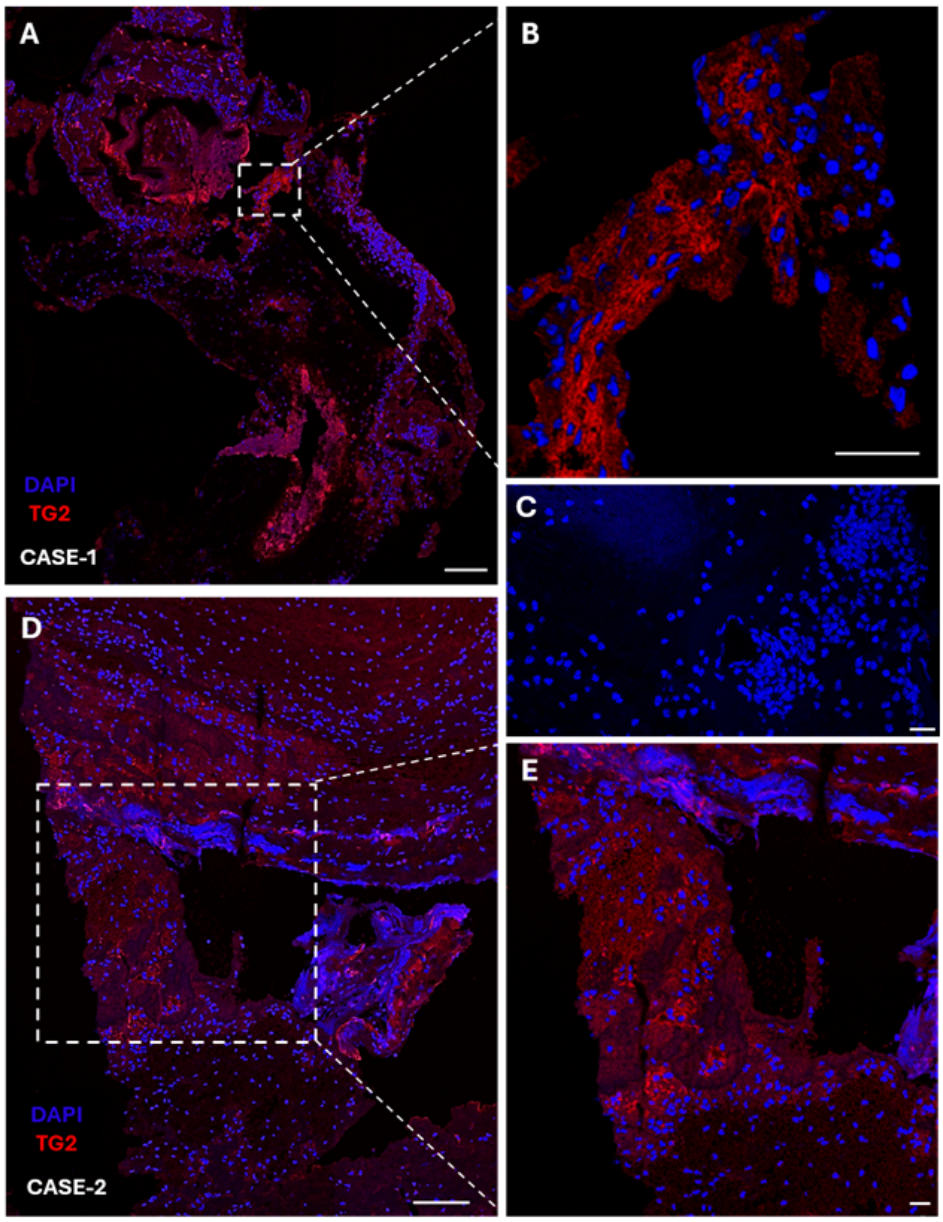
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Figure 2

Figure 2

TG2 in human coagulation

(a) Expression of TG2 protein, stained with CUB7402 antibody (red), in model thrombus from wt mice. TG2, stained clot; c, control. Fresh-frozen thrombi were prepared by snap freezing in liquid nitrogen and sectioned. The APAAP method was used to stain thrombi for TG2 with CUB7402 monoclonal antibody.

Control slides, in which the primary isotype antibody was omitted and replaced with buffer or mouse IgG_1 isotype, were included. Bar = 20 mm. **(b)** Inhibition of cross-linking in human thrombi. Thrombi were formed from normal human blood (CTR), ± 1 mM GTPgS, ± 1 mM R283 and lysis by tPA was monitored. Blood was collected from normal healthy well informed volunteers with their consent into 0.1 vol of 0.13M trisodium citrate. GTPgS was incorporated into thrombi at a final concentration of 1mM, a concentration shown to fully inhibit TG2. The TG active site specific inhibitor 1,3-dimethyl-4,5 diphenyl-2 [2 (oxopropyl) thio]imidazolium inhibitor (R283) was added at 1mM final concentration. **(c)** Thrombi were formed from normal human blood (CTR), ± 1 mM GTPgS, ± 1 mM R283 and lysis by tPA was monitored, like in (b) but at different times, comparing clots from control mice with or without the TG active site specific inhibitor 1,3-dimethyl-4,5 diphenyl-2 [2 (oxopropyl) thio]imidazolium inhibitor (R283).



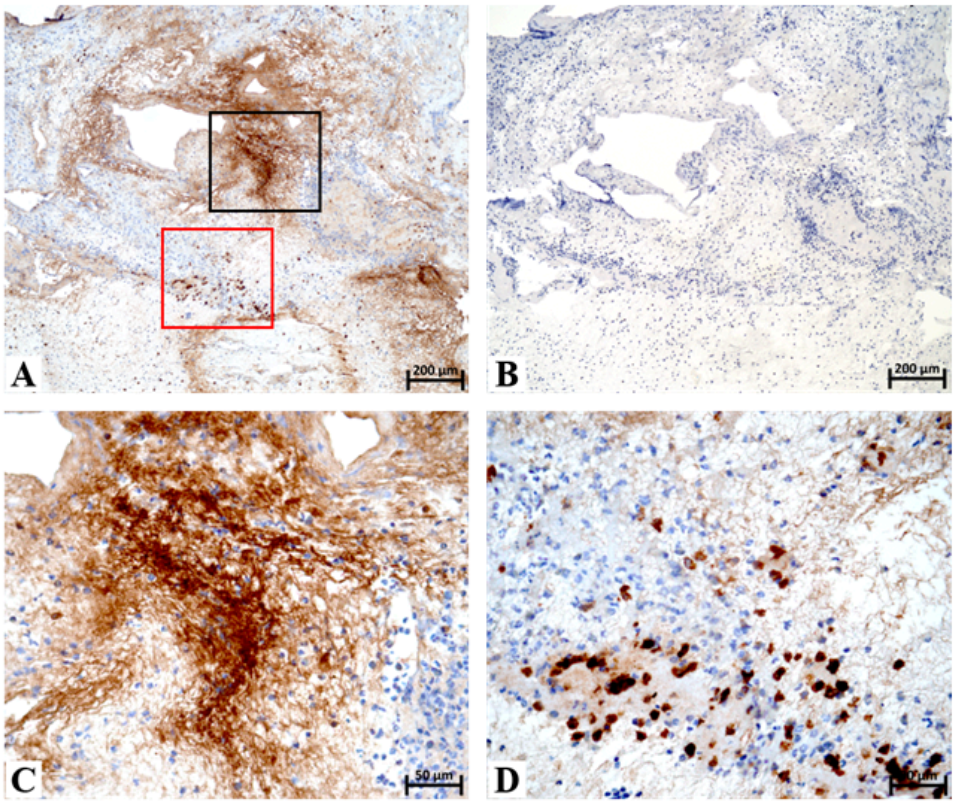
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Figure 3

Figure 3

TG2 in human thrombi from brain stroke patients.

(**A and D**) Large immage of immunofluorescence staining showing TG2 expression (red) in case 1 and case 2 of human thrombus. (**B)**High-Magnifications and 3D super resolution of indicated (white square) TG2 positive areas of the case 1 thrombus. (**C**) Negative control of the immunofluorescence stainig of

the case1 obtainined by incubating tissue slides only with alexa fluor 647 secondary antibody togheter with DAPI, without primary antibody. (**D**) High-Magnifications of indicated (white square) TG2 positive area of the case 2 thrombus . Scale bar is 100 µm (panels A, D), 50 µm (panel B) or 20 µm (panels C and E)..

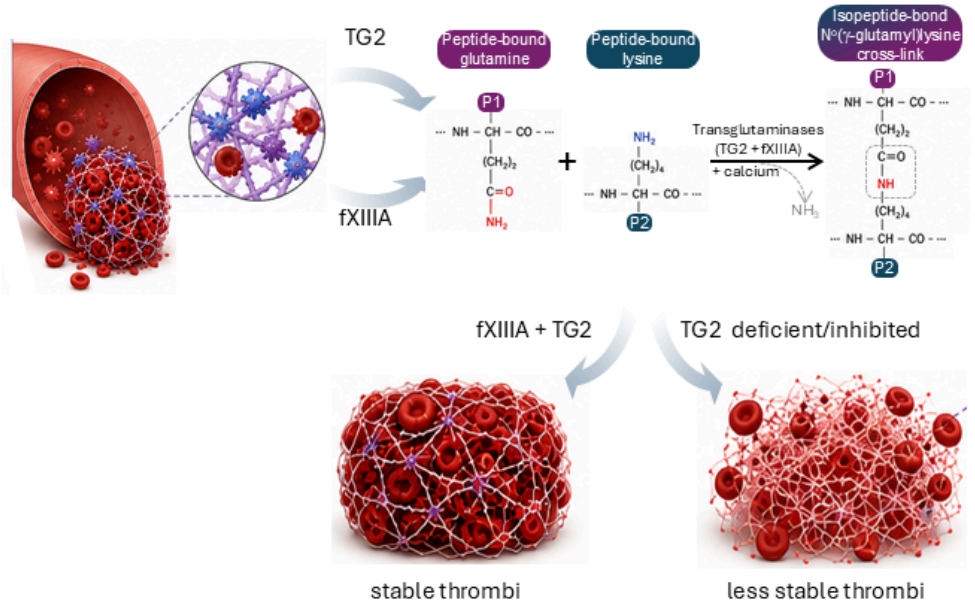


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Figure 4

Figure 4

TG2 expression in human thrombi.

(a) Representative immunohistochemical staining showing TG2 expression in a human thrombus, see Table 1 for clinical details. TG2 positivity is observed in both the extracellular compartment (black square) and blood cells (red square). **(b)** Negative control section processed without primary antibody incubation. **(c)** Higher-magnification of the black square in panel A, showing intense TG2 signal within the extracellular fibrin-rich compartment. **(f)** Higher-magnification of the red square in panel A, highlighting several TG2-positive monocyte/macrophage cells within the thrombus. Scale bar is 200 mm (panels A, B) or 50 mm (panels C,D).



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Figure 5

Figure 5

TG2 contributes to Haemostasis.

During the formation of the clot, fibrin as well as other proteins becomes cross-linked by factor XIIIA, providing rigidity and stability to the clot, including a tight regulation of fibrinolysis. This function is provided by clotting factor XIIIA, that belongs to the family of transglutaminases. In addition to factor

XIIIA, TG2, abundant in erythrocytes, catalizes e-(g-glutamil) lysine cross-links in fibrin and associated proteins. Evidence from TG2-deficient mice and from human thrombi treated with a specific TG2 inhibitor demonstrates that loss or inhibition of TG2 results in less stable clots. Therefore, we propose that TG2 is able to contribute to the cross-linking of fibrin and associated proteins, promoting clot stability and modulating fibrinolysis. These findings support the involvement of TG2 as an additional supporting factor in the blood coagulation cascade.