Novel Function of Tenascin-C, a Matrix Protein Relevant to Atherosclerosis, in Platelet Recruitment and Activation Under Flow

Mathieu Schaff, Nicolas Receveur, Catherine Bourdon, Virginie Wurtz, Cécile V. Denis, Gertraud Orend, Christian Gachet, François Lanza, Pierre H. Mangin

Objective—To prevent platelet adhesion with components exclusively present in atherosclerotic plaques, such as tenascin-C (TN-C, an attractive candidate because this matrix protein is preferentially found within atheroma), as an effective and safe antithrombotic strategy.

Methods and Results—We show that platelets efficiently adhere to TN-C under both static and flow conditions. Videomicroscopy revealed unique behavior under flow conditions, with platelets exhibiting stationary adhesion to TN-C; in contrast, platelets rolled over von Willebrand factor and detached from fibrinogen. Platelet interaction with TN-C was predominantly supported by integrin αIIbβ1 under static conditions; under high shear, it was dependent on both the α2β1 integrin and the glycoprotein Ib-IX complex. Integrin αIIbβ1 appeared to play a secondary role but only at low shear rates. A glycoprotein Ib-IX–dependent interaction was indirect, relying on von Willebrand factor, and increased as a function of wall shear rate. von Willebrand factor bound directly to TN-C, as shown by ELISA and coimmunoprecipitation, suggesting that it acts as a bridge between TN-C and platelets. The adhesion of platelets to TN-C triggered their activation, as demonstrated by a shape change and increases in intracellular calcium level.

Conclusion—This study provides evidence that TN-C serves as a novel adhesive matrix for platelets in a context that is relevant to atherothrombosis.

Key Words: blood flow ■ platelets ■ receptors ■ thrombosis ■ tenascin-C

The central role of platelets in arterial thrombosis renders them attractive targets for antithrombotic drugs. Clopidogrel and integrin αIIbβ3 blockers, 2 antiplatelet agents widely used in clinical practice, have greatly reduced cardiovascular-associated death, however, because they directly target the hemostatic function of platelets, their use is linked to an increased bleeding risk. The development of more selective antithrombotic drugs causing minimal perturbation of hemostasis could pave the way to new and more effective strategies in the treatment of ischemic events. This task is challenged by the fact that the main cellular and molecular events implicated in thrombosis are also those regulating hemostasis. Nevertheless, differences exist between these 2 conditions, such as the rheological microenvironment and the nature of the vascular material exposed to platelets. Thrombosis occurs in advanced atherosclerotic arteries that present abnormal luminal narrowing or stenosis inducing local disturbance of blood flow.1 In addition, the marked inflammation and active tissue remodeling found in atheroma profoundly modify the composition of the subendothelium. Atherosclerotic plaques are rich in highly reactive platelet-adhesive materials, such as collagens or lipids, and overexpress extracellular matrix proteins that are essentially absent from the healthy vessel wall. The identification of platelet-adhesive proteins preferentially expressed in diseased vessels could provide novel targets for more selective antithrombotic therapies.

In this context, tenascin-C (TN-C) appears to be an interesting candidate because it is highly expressed in mouse and human advanced atherosclerotic plaques, whereas it is much less present in healthy vessels.2–4 By using a TN-C–specific antibody, we confirmed a previous observation that the plaques located in the carotid arteries of 30-week-old apolipoprotein E–deficient mice present strong expression of TN-C (supplemental Figure I; all supplemental material available online at http://atvb.ahajournals.org).3 In contrast, we detected a much weaker TN-C signal in the carotid arteries of wild-type mice and in the healthy portions of apolipoprotein E–deficient vessels, suggesting that this protein could be more relevant to pathological thrombosis than to normal hemostasis. The role of TN-C in atherosclerosis is not

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From Inserm U949 (M.S., N.R., C.B., V.W., C.G., F.L., and P.H.M.), Strasbourg, France; the Université de Strasbourg (M.S., N.R., C.B., V.W., C.G., F.L., and P.H.M.), Strasbourg, France; the Etablissement Français du Sang-Alsace (M.S., N.R., C.B., V.W., C.G., F.L., and P.H.M.), Strasbourg, France; Inserm U770 (C.V.D.), Le Kremlin–Bicêtre, France; Université de Paris-Sud (C.V.D.), Le Kremlin–Bicêtre, France; Inserm U682 (G.O.), Strasbourg, France; Université de Strasbourg (G.O.), Strasbourg, France; and the Department of Molecular Biology (G.O.), CHRU Strasbourg, Strasbourg, France.
Correspondence to Pierre H. Mangin, PhD, Inserm UMR-S949, Etablissement Français du Sang-Alsace 10, rue Spielmann, BP 36, 67065 Strasbourg CEDEX, France. E-mail pierre.mangin@efs-alsace.fr
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clearly defined, but this matrix protein could participate in plaque destabilization. Because TN-C has been localized close to the ruptured areas of advanced plaques, it could be exposed to circulating blood and platelets on plaque rupture.

TN-C is a large glycoprotein (GP) found in the extracellular matrix of vertebrate organisms that modulate cellular responses. It is produced at high levels during embryogenesis but is almost absent in adulthood. However, this protein is strongly reexpressed in tissues undergoing remodeling, such as during wound healing or pathological states, including tumor progression and inflammation. TN-C has been implicated in the modulation of cell adhesion, migration, growth, and apoptosis. Although often reported as an antiadhesive protein, TN-C has been described to support the adhesion of endothelial cells through integrins, including α5β1 and α3β1.

The aim of our study was to determine whether TN-C supports platelet adhesion and activation and, thus, could represent an interesting new target for a potentially safer antithrombotic strategy. To investigate the role of TN-C in platelet adhesion and activation, we used both static and dynamic assays. Our study identified a novel role of TN-C as an efficient adhesive and activating protein for platelets. The platelet–TN-C interaction was mediated mainly by integrin αIIbβ3 and the GPIb-IX complex and less by integrin α6β1. GPIb-IX–mediated adhesion occurred only under flow conditions, was indirect, and depended on the binding of von Willebrand factor (VWF) to TN-C. Finally, the adhesion of platelets to TN-C resulted in their activation, as demonstrated by shape change of the cells and increases in intraplatelet Ca2+ levels.

Methods

In Vitro Static and Dynamic Adhesion Assays
Glass coverslips were coated with 100 μg/mL TN-C, FGN, or VWF for 2 hours at room temperature and blocked with PBS plus 10-mg/mL human serum albumin for 60 minutes. We checked that the protein concentrations used for coating gave maximal platelet adhesion. Washed human platelets in Tyrode buffer were allowed to adhere to the coated surfaces for 20 or 40 minutes at 37°C, and the adherent cells were processed as previously described. Flow-based experiments were performed using hirudinated whole blood, 100 U/mL, from human or mouse, as previously described.

Analysis of Cytosolic Ca2+ Fluxes and In Vitro TN-C Binding Assay
Platelet intracellular Ca2+ changes were monitored using a quantitative dual-dye ratiometric assay according to a modification of a previously published method. ELISA 96-well plates were coated with 1- or 5-μg/mL TN-C overnight at 4°C and blocked with PBS plus 20-mg/mL BSA for 1 hour at 37°C. Increasing concentrations of VWF in blocking solution were added and incubated for 2 hours at 37°C. To detect bound VWF, a horseradish peroxidase–conjugated anti-VWF antibody was added (0.4 μg/mL in blocking solution) for 1 hour at room temperature, and the plates were then incubated with O-phenylenediamine. After 5 minutes of substrate conversion, the reaction was discontinued with 50 μL of 3-mol/L H2SO4 and the plates were read at 490 nm.

Immunoprecipitation
Purified TN-C and VWF were coincubated (1 μg of TN-C or VWF with 6.6 μg of VWF or 3.8 μg of TN-C, respectively) in 50 μL of lysis buffer for 2 hours at 4°C. The protein samples were then incubated with 1 μg of an anti–TN-C, anti-VWF, or irrelevant IgG antibody for 1 hour at 4°C, followed by 50 μL of 50% (wt/vol) protein G-Sepharose beads for 1 hour at 4°C. Immune precipitation experiments and Western blotting were performed as previously described.

Statistical Analyses
All values are reported as mean±SEM unless otherwise indicated. Data were compared by 1-tailed paired t tests when studying the effect of blocking monoclonal antibodies and by 1-tailed unpaired t tests in experiments with mice. Differences were considered significant at P<0.05, and all tests were performed using commercially available software (Prism; GraphPad, La Jolla, Calif).

Results
Washed Platelets Adhere to Immobilized TN-C Under Static Conditions
To determine whether TN-C can interact with platelets, we first evaluated the capacity of this matrix protein to support the adhesion of washed human platelets under static conditions. As shown in Figure 1A, numerous platelets adhered to
immobilized TN-C. Interestingly, the number of adherent cells after 40 minutes (3513 ± 307 platelets/mm²; n = 3) was similar to that observed on FGN (4296 ± 681 platelets/mm²; n = 3), which efficiently binds platelets under static conditions (Figure 1B). In contrast, 4.3 times more platelets adhered to TN-C than to VWF (812 ± 123 platelets/mm²; n = 3), which is a weak adhesive protein under static conditions but supports efficient platelet adhesion under flow or in the presence of mediators such as botrocetin (8228 ± 508 platelets/mm²; n = 3) (Figure 1B). These results indicated that platelets adhere to TN-C under static conditions in numbers comparable to those observed on 2 other effective adhesive proteins. Moreover, platelets tended to spread on attachment to FGN or VWF in the presence of botrocetin, reflecting their activation. In contrast, most of them retained the resting discoid morphology after adhesion to TN-C or VWF without botrocetin (Figure 1A).

Platelets Adhere to Immobilized TN-C Under Dynamic Conditions

Next, we explored the capacity of TN-C to support platelet adhesion under flow by perfusing whole blood through glass microcapillaries at wall shear rates found in vessels prone to atherosclerosis, such as the coronary artery (300/second). Real-time differential interference contrast microscopy showed that, after 8 minutes, the number of adherent cells was 1.9 and 6.2 times higher on TN-C (58 050 ± 5253 platelets/mm²; n = 8) than on VWF (30 950 ± 1539 platelets/mm²; n = 6) or FGN (9287 ± 2571 platelets/mm²; n = 8), respectively (Figure 2A). These results pointed to a greater adhesiveness of TN-C compared with VWF or FGN under the shear conditions found in large arteries. Concerning the adhesive behavior, the platelets rolled over VWF (79 ± 2%; n = 4) and rapidly detached from FGN (83 ± 3%; n = 4), whereas most of them attaching to TN-C remained stationary adherent (87 ± 4%; n = 4) (Figure 2B). This different behavior probably explains the enhanced platelet accumulation observed on TN-C compared with VWF or FGN (Figure 2A). Adhesion to TN-C as a function of wall shear rate exhibited a bell-shaped curve, with maximal adhesion at 1000/second (49 790 ± 8629 platelets/mm²; n = 7) (Figure 2C and D). Adhesion to VWF similarly increased up to 1000/second to 1500/second (56 600 ± 2307 platelets/mm²; n = 3) but remained at a plateau level. As previously described, the adhesion of platelets to FGN was inefficient at shear rates greater than 300/second. Remarkably, TN-C was more effective than VWF or FGN in the recruitment of stationary adherent platelets, at least up to 1000/second (Figure 2E).
Platelet Adhesion to TN-C Is Primarily Mediated by Integrin α3β1

Blocking the VWF binding site of GPIb-IX or the FGN binding domain of αIIbβ3 (107±17% and 101±10% of control, respectively; n=3) did not prevent platelet adhesion to TN-C in the static adhesion assay, whereas a pan-blocking anti–β1 antibody significantly reduced it (23±2% of control; n=3; P<0.001) (Figure 3A). The key role of the β1 integrin was also demonstrated in the dynamic adhesion assay, in which 2 anti–β1 antibodies (4B4 and P5D2) profoundly inhibited platelet adhesion to TN-C at both 300/second (by 99.3±0.3% and 79.0±6.0%, respectively) and 1500/second (by 98.4±1.2% and 99.2±0.4%, respectively) (Figure 3B and C).

These results were confirmed in β1-deficient mice presenting a 73±11% (n=5; P<0.01) reduction in the number of adherent platelets to TN-C at 2500/second (Figure 3D). To identify the integrin subtype involved, adhesion was next evaluated in a mouse strain specifically lacking integrin α3β1. In α3β1-deficient platelets, the degree of inhibition was similar to that in β1 deficiency (77±14% reduction relative to wild type; n=4; P<0.05) (Figure 3D), indicating that integrin α3β1 is likely to be the main β1 integrin involved in platelet/TN-C interaction.

Blockade of α3β1, which has been proposed as a TN-C receptor on endothelial cells, only slightly reduced platelet adhesion under flow (by 9±2%; n=3; P<0.05), suggesting a minor role for this integrin (Figure 3B). In contrast, ALMA.12 and AK2, 2 monoclonal antibodies directed against the 45-kDa N-terminal domain of GPIbα, markedly inhibited platelet adhesion to TN-C at 300/second (by 64±10% and 66±17%, respectively; n=3; P<0.05), whereas WM23 against the macroglycopeptide region had no significant effect (77±12% of control; n=3; P=0.09) (Figure 3B).

ALMA.12 reduced platelet adhesion by 95.5±0.9% at 1500/second, indicating that the importance of GPIbα in TN-C adhesion increases with wall shear rate (Figure 3C). In comparison, Integrilin and ReoPro, 2 αIIbβ3 blockers in clinical use, diminished platelet adhesion only under low shear conditions (300/second), with 39±10% and 42±7% inhibition, respectively (n=4; P<0.01) (Figure 3B and C). These findings indicated that the GPIb-IX complex and, to a lesser extent, integrin αIIbβ3 participate in platelet adhesion to TN-C under dynamic, but not static, conditions.

GPIb-Dependent Adhesion of Platelets to TN-C Depends on VWF

The fact that monoclonal antibodies inhibiting VWF binding to GPIbα diminished platelet adhesion to TN-C suggested that VWF could serve as an intermediate ligand. This hypothesis was further supported by the ability of 701, an anti–VWF A1 domain monoclonal antibody blocking GPIbα binding, to reduce platelet adhesion to TN-C by 27±7% at 300/second (n=3, P<0.05) and by 87±7% at 1500/second (n=5, P<0.001) (Figure 4A). Final confirmation of the implication of VWF derived from experiments in VWF-deficient mice,
coprecipitation of TN-C was assessed by SDS-PAGE and Western blotting using a monoclonal anti–TN-C antibody. Purified TN-C was used as a positive control. Data are representative of 4 separate experiments.

VWF Binds to Purified TN-C
To further investigate the capacity of VWF to mediate GPIb-dependent adhesion to TN-C, we determined in an ELISA whether these molecules directly interact with each other. As shown in Figure 4C, soluble VWF bound to immobilized TN-C, but not to BSA, reaching saturation at 5 μg/mL. The interaction was also observed when both molecules were in solution because immunoprecipitated TN-C coprecipitated with VWF and vice versa (Figure 4D). These results demonstrated that VWF interacts with TN-C, thereby supporting a model in which VWF acts as a bridge between TN-C and platelets.

Adhesive Properties of TN-C and VWF Synergize in Platelet Adhesion Under Flow
Because TN-C and VWF were both described to be strongly expressed in atherosclerotic plaques and to more precisely define the functional link between these proteins, we explored the capacity of a combined TN-C–VWF matrix to support platelet adhesion. By using blood from VWF-deficient mice to prevent the participation of plasmatic VWF, we observed a marked enhancement of platelet adhesion to the mixed proteins (17 100±3051 platelets/mm²; n=3; after 2 minutes) at 3000/second, which was 2.9 and 2.6 times higher than on TN-C (5833±1073 platelets/mm²; n=3; after 2 minutes) or VWF (6533±1686 platelets/mm²; n=3; after 2 minutes) alone, respectively (Figure 5). These results demonstrated that TN-C synergizes with VWF to support platelet adhesion under flow.

Activation of Platelets on Adhesion to TN-C
Platelet shape change represents 1 of the first measurable signs on activation and results in the transformation of a discoid and smooth resting cell into a spherical form, extending filopodia and ultimately spreading to a “fried egg”–like morphology. Although the platelets adhering to TN-C under static conditions tended to remain discoid (Figure 1A), most of them became spherical and extended filopodia (82%±4%, n=3) at 300/second, similar to what occurred on VWF (77±2%, n=3) or FGN (75±4%, n=3) (Figure 6A and B).

To explore the capacity of TN-C to induce intracellular signaling, we measured cytosolic Ca²⁺ changes on adhesion of platelets under flow, using a quantitative dual-dye ratio-metric Ca²⁺ assay. As shown in Figure 6C, although individual resting platelets displayed low Ca²⁺ concentrations (<50 nmol/L), most of those adhering to TN-C exhibited pulsatile and high-frequency Ca²⁺ spikes (Figure 6D and E), ranging...
between 50 and 1650 nmol/L (maximal increase, 363±55 nmol/L; n=50). Platelets adhering to FGN displayed similar variable Ca$^{2+}$ responses (maximal increase, 383±61 nmol/L; n=40); they were less important on VWF (maximal increase, 167±31 nmol/L; n=40). The increased fluorescence was the result of enhanced cytosolic Ca$^{2+}$ because pretreating platelets with the Ca$^{2+}$ chelator DM-BAPTA-AM reduced the mean signal by 96±1% (n=30) (Figure 6C). Altogether, these results showed that the adhesion of platelets to TN-C under flow conditions leads to their activation.

**Discussion**

The studies reported herein identify a novel role of TN-C in platelet physiology and suggest that this extracellular matrix protein could play an important part in atherothrombosis. We provide evidence that TN-C supports efficient platelet adhesion under static and dynamic conditions of up to 1500/s second. Contrary to VWF and FGN, TN-C promoted the stationary adhesion of platelets under flow. We showed that the interaction of platelets with TN-C is a complex process involving at least 3 receptors (ie, integrin $\alpha_2\beta_1$, integrin $\alpha_{IIb}\beta_3$, and the GPIb-IX complex). Added complexity arises from the fact that platelet attachment to TN-C is in part indirect and occurs through VWF binding. Once attached to TN-C, the platelets become activated, as attested by their shape change and increased intracellular Ca$^{2+}$ levels.

The data from static adhesion experiments indicated that a platelet surface $\beta_1$ integrin bound to TN-C. This is in agreement with previous reports indicating that other cell types, such as endothelial and tumoral cells, interact with TN-C through $\beta_1$ integrins, in particular $\alpha_2\beta_1$, $\alpha_6\beta_1$, and $\alpha_{IIb}\beta_3$. Platelets contain 3 $\beta_1$ integrins ($\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, and $\alpha_{IIb}\beta_1$) that bind to collagen, fibronectin, and laminin, respectively. Integrons $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_1$ have not been described in platelets; this was confirmed in Western blotting and RT-PCR assays (data not shown), thereby pointing to $\alpha_{IIb}\beta_3$ as the likely candidate for platelet/TN-C interaction. Experiments with genetically modified mice confirmed this hypothesis, providing the first report that another matrix protein than collagen interacts with platelet $\alpha_{IIb}\beta_3$. Although we cannot formally exclude a role of $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, the identical degree of inhibition of platelet adhesion to TN-C in $\alpha_2$- and $\beta_3$-deficient mice suggests that $\alpha_{IIb}\beta_3$ is the only $\beta_1$ integrin involved. We did not formally demonstrate a direct interaction between $\alpha_{IIb}\beta_3$ and TN-C. However, this was strongly suggested because isolated platelets pretreated with prostaglandin E$_1$ to prevent release of adhesive proteins stored in $\alpha$-granules still efficiently adhered to purified TN-C.

The blockade of $\alpha_{IIb}\beta_3$ at high shear resulted in a marked reduction in platelet adhesion, with many fewer platelets attaching to TN-C. This result was surprising because integrins are not usually described as contributing to platelet recruitment under high shear conditions as the result of their slow association rate.$^{13}$ Moreover, the few platelets adhering under $\alpha_{IIb}\beta_3$ blockade no longer remained stationary but rapidly detached, suggesting that $\alpha_{IIb}\beta_3$ also participates in supporting stationary adhesion to TN-C. This is in agreement with the well-recognized role of integrins in stable bond formation as the result of their slow dissociation rates.$^{12}$ Two different antibodies blocking collagen binding to $\alpha_{IIb}\beta_3$ did not prevent adhesion to TN-C (supplemental Figure II), indicating distinct binding sites for TN-C and collagen.

Because antibodies preventing VWF binding to GPIb$\alpha$ inhibited platelet adhesion to TN-C, GPIb$\alpha$ participation was presumably indirect, requiring the prior binding of VWF to TN-C. An analogous mechanism has also been described for platelet adhesion to collagen, which is, in part, mediated through VWF.$^{14}$ One difference was that, although human platelets adhered to VWF even at greater than 1500/s second, this was no longer the case for TN-C. This could be the result of a suboptimal amount of TNC-bound plasmatic VWF. Interestingly, normal mouse platelets still efficiently adhered to TN-C at shear rates of up to 2500/s second. The ability of mouse platelets to adhere at higher shear rates when compared with human platelets has also been described on a collagen matrix.$^{14,15}$ This difference could arise from the smaller size of mouse platelets, which reduces the drag forces applied to them, or from different affinities of the receptor-ligand interactions between the 2 species.

Integron $\alpha_{IIb}\beta_3$ is also involved in platelet adhesion to TN-C. This interaction could be indirect through VWF, a known $\alpha_{IIb}\beta_3$ ligand, or direct because TN-C contains a region presenting strong homology with the $\gamma$ chain of FGN$^{16}$ and an RGD sequence in its third fibronectin type III domain.$^{17}$ Unlike GPIb$\alpha$, the importance of $\alpha_{IIb}\beta_3$ decreased with wall shear rate, which could be because of a low resistance to tensile stress that does not allow $\alpha_{IIb}\beta_3$ to withstand high shear. $\alpha_{IIb}\beta_3$, the second platelet $\beta_3$ integrin, mediates endothelial and tumoral cell adhesion to TN-C$^{15,16}$ but is not critical in platelet/TN-C interaction. This could be explained by its low expression (250 receptors per platelet) compared with integrin $\alpha_{IIb}\beta_3$ (80,000 receptors per platelet).

Platelets adhering to TN-C became activated as indicated by their shape change and intracellular Ca$^{2+}$ oscillations,
suggesting that the role of TN-C goes beyond its ability to recruit platelets. Because the GPIb/VWF interaction triggers filopodia extension and transient Ca\(^{2+}\) events,\(^8\) it could be implicated in the TN-C response. In addition, outside-in and IIb/III\(a\) signaling also induce morphological changes and elevated Ca\(^{2+}\) oscillations in platelets.\(^8,18\) Given that Ca\(^{2+}\) was measured in the absence of plasma VWF, it points to a likely role of IIb/III\(a\) in our assay conditions. This would agree with the similar Ca\(^{2+}\) oscillations observed herein on TN-C and in platelets adhering to the IIb/III\(a\)-specific GFOGER peptide.\(^18\) Future studies are required to determine the contribution of the different receptors and of the additional amplification by soluble agonists, such as ADP or thromboxane A\(_2\), which are released by activated platelets.

Platelets efficiently adhered to TN-C across a wide range of wall shear rates, with maximal adhesion at approximately 1000/second to 1500/second. VWF\(^12\) and thrombospondin-1 are the only adhesive proteins known to support human platelet adhesion under high shear; the other matrix proteins, including FGN and fibronectin, do not display this property. The shear conditions under which platelets interact with TN-C are of pathological relevance because 1000/second represents the mean peak wall shear rate found in diseased carotid arteries presenting 78% stenosis.\(^19\) Given that 60% stenosis is clinically rated as significant and 70% is rated as severe,\(^20\) the TN-C exposed on plaque erosion or rupture could participate in platelet recruitment. Moreover, even in the case of severe stenosis (>90%), local regions of low shear (<1000/second) extend into the downstream expansion zone,\(^19\) where TN-C could also participate in the first step of thrombus growth.

Pharmacologically, blocking platelet–TN-C interaction could inhibit the initial stage of platelet recruitment and activation on a ruptured plaque and thereby potentially prevent excessive thrombosis. Because TN-C is almost absent in healthy vessels,\(^2\) agents blocking platelet–TN-C interaction might be expected to be highly selective for thrombosis and would not affect normal hemostasis. This possibility is supported by the fact that TN-C-deficient mice present a normal tail bleeding time (M.S., unpublished data, 2010). From a clinical point of view, an agent that prevents recruitment of platelets to a site of plaque rupture without affecting hemostasis would, in theory, provide a wide therapeutic window with a minor risk of bleeding.
Based on the present findings, the following working model is proposed. On erosion or rupture of an atherosclerotic plaque, TN-C present at high levels will become exposed to circulating blood, bind plasmatic VWF, and capture circulating platelets through interaction with the GP Ib-IX complex. Exposed TN-C will also recruit platelets through direct interaction with integrin α2β1. Stable adhesion will be established through integrins, primarily α2β1 and αIIbβ3, that bind to VWF and/or TN-C. Finally, engagement of these different receptors will induce platelet activation, resulting in shape change and increase in intracellular Ca2+ levels.

The full implication of TN-C in thrombosis and its importance with respect to other pathways remain to be clearly delineated. The observation that platelets do not form aggregates on TN-C suggests that it might be much less thrombogenic than highly reactive plaque components, such as collagen, lipid derivatives, or tissue factor. Rather, we postulate that TN-C could provide additional contacts increasing platelet–vessel wall interaction and platelet activation on plaque rupture. In particular, the fact that platelet recruitment under flow was greatly enhanced on a mixed TN-C–VWF substrate compared with individual proteins strongly suggests that TN-C could act in combination with other platelet-reactive components to promote full integrin αIIbβ3 activation and efficient thrombus growth.

In conclusion, this previously unrecognized role of TN-C in platelet adhesion and activation could be of great significance in the context of the shear conditions encountered in arterial thrombosis. This study provides an interesting candidate in the context of the shear conditions encountered in arterial thrombosis. We thank Monique Fréaud for animal care; Isabelle Gasser and Stéphanie Magnenat for technical assistance, and Juliette Mulvihill for editorial help.

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**References**

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SUPPLEMENT MATERIAL

Detailed Methods

Materials

Prostaglandin E₁ (PGE₁), prostaglandin I₂ (PGI₂), bovine serum albumin (BSA), fatty acid-free human serum albumin (HSA), TRITC (tetramethylrhodamine isothiocyanate)-phalloidin and human FGN were purchased from Sigma-Aldrich (St. Louis, MO) and protein G-Sepharose from GE Healthcare (Little Chalfont, United Kingdom). 1-paraformaldehyde (PFA) was from Electron Microscopy Sciences (Hatfield, PA) and O-phenylenediamine from Thermo Scientific (Waltham, MA). Human VWF was isolated from factor VIII concentrates (EFS-Alsace, Strasbourg, France) according to the method of Toti et al.¹ Botrocetin was purified from snake venom (Latoxan, Valence, France) as described previously.² Experiments were performed with different batches of human TN-C from the U251 glioma cell line (Millipore, Billerica, MA). Western blotting, silver and Coomassie blue stainings and two different techniques of mass spectrometry (MALDI-MS and LC-MS/MS) were used to ensure that very pure TN-C was used. This TN-C was structurally related to that found in atherosclerotic vessels.³,⁴ In addition, similar results were obtained with a different source of TN-C (AbD Serotec, Raleigh, NC; data not shown). Apyrase was purified from potatoes as previously described.⁵ DM-BAPTA-AM (5,5’-dimethyl-1,2-bis(O-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid-tetra(acetoxymethyl) ester) and the indicators Oregon Green 488 BAPTA-AM-4 and Calcein red-orange-AM were from Molecular Probes (Eugene, OR). Acid citrate dextrose (ACD) solution was obtained from Bioluz (St-Jean-de-Luz, France), hirudin from Transgene (Illkirch-Graffenstaden, France) and heparin from Sanofi-Aventis (Paris, France). The integrin α₁β₃ antagonist eptifibatide (Integrilin®) was purchased from Schering-Plough (Kenilworth, NJ). The monoclonal antibody (mAb) DB7 against TN-C was from Millipore, horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) from Jackson Lab (Bar Harbor, ME) and the HRP-conjugated anti-VWF
antibody from Dako (Glostrup, Denmark). Mouse monoclonal blocking antibodies were as follows: anti-human integrin β1, 4B4 (Beckman Coulter, Fullerton, CA) and P5D2 (Abcam, Cambridge, United Kingdom); anti-human GPIbα, AK2 (Abcam) and ALMA.12 (produced in our laboratory); anti-human integrin αvβ3, LM609 (Millipore); anti-human integrin αⅡbβ3, chimeric mAb 7E3 Fab fragments abciximab (ReoPro®) (E. Lilly, Indianapolis, IN); anti-human VWF A1 domain, clone 701 kindly provided by Dr. C. Denis. Other antibodies used were: WM23, against the macroglycopeptide region of GPIbα (Pr. S. Jackson, Monash University, Melbourne, Australia) and MOPC-21, a mouse IgG1κ isotype control (Biolegend, San Diego, CA).

Mice

Mice were backcrossed onto the C57BL/6 background for a minimum of 6 or 12 generations and were maintained in the animal facilities of the Etablissement Français du sang-Alsace. VWF-deficient (VWF−/−) mice were from Dr. C. Denis, β1-null (β1−/−) mice from Dr. R. Fässler (Max Planck Institute, Martinsried, Germany) and α2-null (α2−/−) mice from Dr. B. Eckes (University of Cologne, Cologne, Germany). A WT littermate was purchased from Charles River (Wilmington, MA).

Blood Collection and Preparation of Washed Platelets and Red Blood Cells

Blood was drawn from the abdominal aorta in 8-week-old mice (about 5 animals for each condition) anesthetized intraperitoneally with a mixture of xylazine (20 mg per kg body weight, Rompun®, Bayer, Leverkusen, Germany) and ketamine (100 mg per kg body weight, Imalgene 1,000®, Merial, Lyon, France). All experiments conformed to the French legislation for animal experimentation and followed the recommendations of the Guide for the Care and Use of Laboratory Animals.

Human blood was collected from healthy volunteers who had not taken any antiplatelet medication in the preceding 2 weeks. Platelets were washed using ACD-anticoagulated whole blood as previously described. Briefly, the platelet-rich plasma (PRP) was obtained by centrifugating blood at
250 x g for 16 min. Following a 10 min rest period, the PRP was centrifuged at 2,200 x g for 16 min. The platelet poor plasma was then removed by aspiration and the pelleted platelets were gently resuspended in an equal volume of isotonic Tyrode’s Buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, 5 mmol/L Hepes, 3.5 mg/mL HSA, 5.5 mmol/L glucose, pH 7.3) supplemented with 10 U/mL heparin and 0.5 µmol/L PGI₂. Following another 10 min rest period, 0.5 µmol/L PGI₂ was added to the platelets before centrifugation at 1,900 x g for 8 min. This step was performed twice and platelets were finally resuspended to a final concentration of 3 x 10⁸ platelets/mL in Tyrode’s Buffer supplemented with 0.02 U/mL apyrase. Washed platelets were kept at 37°C for 30 min prior to experimentation in order for apyrase activity to decay and studies were achieved within 6 hours after blood collection. To prepare reconstituted blood, red blood cells (RBCs) were obtained after centrifugating whole blood at 250 x g for 16 min. The PRP and leukocyte layers were removed and the RBCs were washed twice in Tyrode’s buffer and treated with 0.02 U/mL apyrase, prior to reconstitution with autologous washed platelets (50% (v/v)) at a final concentration of 250 x 10⁶ platelets/mL.

**Static Adhesion Assay**

Glass coverslips were coated with 100 µg/mL TN-C, FGN or VWF for 2 hours at RT and blocked with PBS-10 mg/mL (1%) HSA for 60 min. We checked that the protein concentrations used for coating gave maximal platelet adhesion. Human platelets (4.5 x 10⁶/coverslip) in Tyrode's buffer were allowed to adhere to the coated surfaces at 37°C. After 20 or 40 min, non-adherent platelets were washed away and adherent cells were fixed with PBS-40 mg/mL (4%) PFA and imaged by differential interference contrast (DIC) microscopy. In the case of VWF surfaces, the modulator botrocetin (2 µg/mL) was added since this matrix supports weak adhesion in the absence of fluid shear. Where indicated, the platelets were pretreated for 20 min with PGE₁ (10 µmol/L) to keep them in a resting state and prevent granule secretion.
In Vitro Flow-Based Adhesion Assay

Rectangular glass microcapillaries (VitroCom, Mountain Lakes, NJ) were coated with 100 µg/mL TN-C, 300 µg/mL FGN or 100 µg/mL VWF overnight at 4°C and blocked with PBS-10 mg/mL (1%) BSA for 30 min at RT. Concerning the flow experiments in which a double matrix was used, we have preliminarily determined the lowest concentrations of VWF (20 µg/mL) and TN-C (100 µg/mL) providing maximal platelet adhesion. Hirudinated (100 U/mL) whole blood or reconstituted blood was perfused through the coated capillaries with a syringe pump (Harvard Apparatus, Holliston, MA) at 37°C and at various flow rates as previously described. Platelet adhesion was observed in real time under an inverted Leica DMI 4000 B microscope (Leica Microsystems, Wetzlar, Germany) using a 63x, 1.4 numerical aperture oil objective and a DIC technique. Images were acquired with a Photometrics charge-coupled device (CCD) camera (CoolSNAP HQ Monochrome, Photometrics, Tucson, AZ) and analyzed off-line using Metamorph software version 7.6 (Molecular Devices, Downingtown, PA). Platelet adhesive behavior was analyzed frame by frame (15 frames/s) and classified as (i) rolling when platelets constantly moved over the surface of the matrix, (ii) stationary adhesion when they did not move more than one half of a single cell diameter over a 10 s period, and (iii) detaching when detachment occurred within 2 s following the initial contact.

Morphologic Analyses

Hirudinated (100 U/mL) whole blood was perfused through glass microcapillaries coated with TN-C (100 µg/mL), FGN (300 µg/mL) or VWF (100 µg/mL) for 3 min at 300 s⁻¹, followed by washing with PBS at 300 s⁻¹ for 2 min. Adherent platelets were observed by DIC microscopy (Leica DMI 4000 B, 63x/1.4 oil objective). Change in cell shape was defined as the transformation of a resting discoid platelet into an activated spherical cell with filopodial projections more than 0.2 µm in length and a flattened “fried egg”-like morphology.
Analysis of Cytosolic Ca\textsuperscript{2+} Fluxes

Platelet intracellular Ca\textsuperscript{2+} changes were monitored according to a modification of a previously published method.\textsuperscript{12} Briefly, human platelets resuspended at 500 x 10\textsuperscript{6}/mL after the first washing step were simultaneously loaded with the membrane-permeating non ratiometric Ca\textsuperscript{2+} indicator dye Oregon Green 488 BAPTA-AM-1 (4 µmol/L) and the morphological dye Calcein red-orange-AM (2.0 µmol/L) for 30 min at 37°C. The dye-loaded platelets were washed twice and finally resuspended in Tyrode’s buffer containing apyrase (0.02 U/mL). These platelets were incubated with either vehicle (1:1,000 DMSO) or DM-BAPTA-AM (50 µmol/L) for 10 min and reconstituted with 50% (v/v) autologous packed RBCs at 250 x 10\textsuperscript{6} platelets/mL, prior to perfusion over TN-C (100 µg/mL), FGN (300 µg/mL) or VWF (100 µg/mL) at 300 s\textsuperscript{-1}. The increases in platelet and Ca\textsuperscript{2+}-dependent fluorescence intensity upon adhesion of the cells were measured in the ranges 572-700 nm and 495-535 nm, respectively, by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems) (1.85 frames/s for 8 min). The mean fluorescence ratio R\textsubscript{max} was determined from the signal of washed platelets preincubated with A23187 (5 mmol/L) + Ca\textsuperscript{2+} (10 mmol/L) and allowed to adhere to glass coverslips (50 platelets per experiment), while R\textsubscript{min} was calculated from that of platelets preincubated with DM-BAPTA-AM (70 µmol/L) + EGTA (ethyleneglycoltetraacetic acid) (2 mmol/L) and allowed to adhere to 10 mg/mL (1%) BSA-coated coverslips (50 platelets per experiment). The ratio of signal intensity between the two dye channels was converted to intracellular Ca\textsuperscript{2+} concentrations as described previously (Leica TCS SP5 LASAF software).\textsuperscript{12} The estimated Ca\textsuperscript{2+} concentration is indicated relative to the zero point set by DM-BAPTA-AM and EGTA Ca\textsuperscript{2+} chelation. Basal Ca\textsuperscript{2+} levels were determined by measuring the fluorescence ratio (mean ratio=0.6, n=45) in platelets adhering to non-reactive 10 mg/mL (1%) BSA-coated coverslips. A specific Ca\textsuperscript{2+} signal was defined as a change in fluorescence ratio of more than two standard deviations (2σ=0.38, n=45) relative to the mean fluorescence ratio in resting platelets and corresponded to peaks ranging over 50 nmol/L.
In Vitro TN-C Binding Assay

Blocking solution composition: 1:1,000 Tween 20 and 1 mg/mL (0.1%) BSA in PBS.

Immunoprecipitation

Lysis buffer composition: 1:100 Triton X-100, 20 mmol/L Tris (tris(hydroxymethyl)aminomethane)-HCl, 5 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 100 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, pH 7.4.

References


**Supplemental Figure Legends**

**Supplemental Figure I.** TN-C is highly expressed in mouse atherosclerotic plaques whereas it is almost absent in healthy vessel wall. Representative confocal images of transversal PFA-fixed cryosections of an atherosclerotic (*ApoE<sup>-/-</sup>* plaque) or healthy portion (*ApoE<sup>-/-</sup> healthy*) of a carotid artery from an ApoE-deficient mouse or of a carotid artery from a wild-type mouse (*WT*). TN-C (left panel) was detected with a specific mAb (TNC1.2, Dr. G. Orend), followed by a Cy5-conjugated secondary antibody (red). Sections were co-labeled with the nuclear marker DAPI (blue) to visualize the vessel wall. Scale bar, 75 µm. *L* indicates lumen.

**Supplemental Figure II.** Antibodies blocking collagen binding to α<sub>2</sub>β<sub>1</sub> do not prevent platelet adhesion to TN-C. Hirudinated human whole blood was preincubated for 10 min with 10 µg/mL of irrelevant mouse IgG (*Control*) or 10 µg/mL of a mAb blocking collagen binding to α<sub>2</sub>β<sub>1</sub> (*P1E6, BHA2.1*) and perfused through TN-C-coated (100 µg/mL) glass microcapillaries at 300 s<sup>-1</sup>. Control experiments were performed to ensure that P1E6 and BHA2.1 inhibited collagen-induced platelet aggregation. Adherent platelets were counted in one random field for each condition after 7 min of perfusion and their number was expressed as a percentage of that in control set to 100%. Results are the mean ± S.E.M. of three separate experiments (*P1E6, p=0.44, NS; BHA2.1, p=0.18, NS).*
Supplemental Figure II

Platelet adhesion (% of control)

- Control
- P1E6
- BHA2.1