INVolvement of a CD47-Dependent Pathway in Platelet Adhesion on Inflamed Vascular Endothelium Under Flow

running title: CD47 and platelet adhesion on inflamed endothelium

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ABSTRACT

Resting platelet adhesion to inflammatory vascular endothelium is thought to play a causal role in secondary thrombus formation or microcirculatory disturbance after vessel occlusion. However, while adhesion receptors involved in platelet/matrix interactions have been extensively studied, the molecular mechanisms involved in platelet/endothelium interactions are incompletely characterized and have been mainly studied under static conditions. Using human platelets or platelets from wild type and CD47-/- mice in whole blood, we demonstrated that at low shear rate, CD47 expressed on human and mouse platelets significantly contributes to platelet adhesion on TNF-α-stimulated vascular endothelial cells. Using the CD47 agonist peptide 4N1K and blocking monoclonal antibodies (mAbs), we showed that CD47 binds the CBD domain of the endothelial TSP-1 inducing the activation of the platelet αIIbβ3 integrin which in turn becomes able to link the endothelial receptors ICAM-1 and αvβ3. Platelet CD36 and GPIbα are involved too since, platelet incubation with blocking mAbs directed against each of these two receptors significantly decreased platelet arrest. As anti-CD47 treatment of platelets did not further decrease adhesion of anti-CD36-treated platelets and, as CD36 is a TSP-1 receptor, this suggests that CD36/TSP-1 interaction could trigger the CD47-dependent pathway. Overall, CD47 antagonists would be of significant clinical importance. (abernard@hermes.unice.fr)
INTRODUCTION

The interaction of platelets with the vascular wall is an important step in hemostasis and the development of thrombotic lesions. While adhesion receptors involved in platelet/subendothelial matrix interactions have been extensively studied\(^1\)\(^-\)\(^5\), those involved in platelet/endothelium interactions and particularly those involved in resting platelets to inflamed vascular endothelium (IVE) are incompletely characterized. However, this phenomenon is thought to occur during the occlusive, thromboembolic, reperfusion and septic complications of atherosclerotic and diabetic vascular diseases\(^6\).

Normally, endothelial cells act as nonthrombogenic surface but, upon inflammatory stimuli (TNF-\(\alpha\), IL-1\(\beta\)...\) endothelial cells acquire a new phenotype allowing resting platelets to adhere. Platelet adhesion follows the general principles of leukocyte extravasation: tethering and rolling on endothelial cells, rapid activation of integrins allowing platelet firm arrest. Several \textit{in vivo} studies clearly demonstrated that platelet rolling on vessel wall mainly involved endothelial P-selectins\(^7\) and PSGL-1 (Platelet P-Selectin Glycoprotein Ligand-1)\(^8\) and GPIb\(\alpha\) (glycoprotein Ib\(\alpha\), CD42b)\(^9\) as counterreceptors on platelets. In addition to the P-selectin-dependent mechanism, a pathway involving the vWF (von Willebrand factor) present on stimulated endothelial cells and the platelet GPIb\(\alpha\) has been described\(^10\). For firm adhesion, the receptors implicated are not yet totally identified. Only few studies have been performed and usually the adhesion molecules involved have been studied either on resting platelets or on activated endothelial cells but rarely on both sides. \textit{In vivo} experiments presented evidence that PECAM-1 (Platelet Endothelial Cell Adhesion Molecule 1, CD31) on endothelial cells may contribute to platelet adhesion at a site of injured but not denuded endothelium\(^11\). In another \textit{in vivo} study, endothelial cells rendered ischemic have been shown to acquire a procoagulant phenotype, characterized by fibrinogen accumulation which promoted resting platelet adhesion. ICAM-1 (intercellular adhesion molecule 1, CD54) was identified as the receptor for fibrinogen on the endothelial side and the integrin \(\alpha\)IIb\(\beta\)3 on the platelet side\(^12\). \textit{In vitro}, it has been shown that endothelial cells undergoing apoptosis become proadhesive for non activated platelets. Platelet \(\beta\)1 integrins were demonstrated to be involved in this interaction but their endothelial ligand is still unknown\(^13\). With human umbilical vein endothelial cells (HUVEC) infected with herpes virus\(^14\) or stimulated with IL-1\(^15\), platelet adhesion was effectively inhibited by antibodies directed respectively against
the vWF secreted by endothelial cells and the endothelial αvß3 integrin (CD51). Therefore integrins and, especially β1 (CD29) and β3 integrins (CD61) are involved in platelet/IVE interactions.

CD47 or IAP (for integrin-associated protein) is a 50 kDa glycoprotein expressed on all mammalian cells which has been initially described as a molecule physically associated to integrins, able to regulate their functions. So far, two ligands of CD47 have been identified: thrombospondin-1 (TSP-1) and Signal Regulatory Proteinsα (SIRPα). In platelets, where CD47 has been described to be physically associated to β1 and β3 integrins, TSP-1/CD47 interaction was demonstrated firstly, to activate the integrin αIIbß3 which resulted in platelet spreading on immobilized fibrinogen and secondly, to activate the integrin α2ß1 involved in the early activation of platelets upon adhesion to collagen. In a human melanoma cell line, the TSP-1/CD47 interaction was shown to activate αvß3 integrin which enhanced cell spreading on vitronectin.

Since CD47 and the three integrins αIIbß3, αvß3 and α2ß1 are expressed on platelets and since TSP-1 and SIRPα are expressed on endothelial cells, we tested the possibility that CD47 contributes to resting platelet adhesion on IVE. Although the capacity of platelets to adhere greatly depends on tensile strengths generated by blood flow and the availability of plasma components, platelet/endothelium interactions have been mainly studied with washed platelets in static assays. We chose to investigate these interactions using whole blood under flow conditions, at low shear rate (100s⁻¹) which simulates blood flow in venous vessels.
MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human and mouse TNF-α, as well as recombinant human ICAM-1 (rhICAM-1) were obtained from R&D Systems (Abingdon, UK). The 4N1K peptide corresponding to the CBD of TSP-1 (KRFYVVMWKK) was purchased from Bachem (Voisins-le-Bretonneux, France) and the control peptide 4NGG (KRFYGGMWKK) from Mimotopes (Paris, France). In some experiments, thrombin was inactivated with 2U/ml hirudin (Sigma-Aldrich, St Quentin Fallavier, France) for 10 min at room temperature.

The monoclonal antibody (mAb) directed against CD47, "B6H12" was obtained from the American Type Culture Collection (ATCC, Rockville, MD) as well as the mAb "1F5" directed against CD20. Murine anti-TSP-1 mAbs "C6.7", and "A2.5" directed respectively against the CBD or the binding site of heparan sulfates on TSP-1 were purchased from NeoMarkers (Union City, CA). Mabs "SE5A5" directed against SIRPα1 and SIRPα2 was a kind gift of Dr. H.-J. Büring (University of Tübingen, Germany). Anti-α2ß1, "BHA2.1"; anti-αvß3, "LM609" directed against RGD binding sites 21 and anti-GPIIßα, "SZ2" which inhibits ristocetin-induced platelet aggregation 21 proceed from Chemicon Inc. (Temecula, CA). The anti-αIIß3, "P2" which inhibits platelet binding to fibrinogen 22, the anti-GPIIßα (clone SZ2), the anti-vWF (clone 4F9), as well as the anti-CD36 mAbs (clone FA6.152) were purchased from Immunotech (Marseille, France). Anti-ICAM-1, "B-H17" was kindly obtained from Dr. J. Widjenes (Diaclone, Besançon, France). The anti-CD31 mAb (clone WM59) was obtained from Pharmingen (San Diego, CA). All these antibodies are mouse IgG1 and blocking antibodies. They were used at 40µg/ml.

Cells and Mice

The transformed human umbilical vein endothelial cell (HUVEC) line EA.hy926 was kindly provided by Dr. Edgell (University of North Carolina, NC) 23 and cultured in DMEM with 1g/l glucose (Gibco Laboratories) supplemented with 20% FCS, 2mM L-glutamine and 10 mM Hepes. HUVEC cells were purchased from BioWhittaker (Emerainville, France) and cultured in the recommended EGM-2 BulletKit medium (BioWhittaker).

C56BL/6 mice deficient in CD47 were generated as previously described 24. Wild type C57BL/6J mice were purchased from IFCA-CREDO (l’Arbresle, France). Mice aged 8-12 weeks were used.
**Blood sampling and platelet preparation**

For human platelet isolation, blood from healthy adult volunteers was obtained by veinipuncture. The volunteers did not take any drugs for the previous 10 days. Blood was drawn into propylene tubes containing heparin (final concentration, 20 U/ml) and centrifuged at 120 g for 25 min at room temperature to pellet erythrocytes and obtain platelet-rich-plasma (PRP). Subsequently, platelets in the PRP containing 5 U/ml apyrase (Sigma-Aldrich) were stained with 0.5 µg/ml of green calcein-acetoxymethyl ester (Molecular Probes, Eugene, OR) in dark. In some experiments, the PRP was then incubated with different mAbs or peptides for 10-15 min at 37°C and whole blood was reconstituted before perfusion in the flow chamber.

For mouse platelet isolation, wild-type or CD47−/− C57BL/6 mice were used as donors. Murine blood was collected from the retroorbital venous plexus. Blood of each mouse was drawn into 2 ml propylene tube containing heparin (final concentration, 20 U/ml). The blood of 10 mice in each group was pooled and centrifuged at 120 g for 25 min at room temperature to pellet erythrocytes and obtain PRP. No labeling of mouse platelets was observed by adding calcein in the PRP, even at a concentration of 5 µg/ml and a dramatic decrease in platelet adhesion happened with washed platelets. Only a weak labeling was observed with mepacrine thus, DIOC6 (3,3’-dihexyloxacarbocyanine iodide, Sigma-Aldrich) in PRP (final concentration, 2 µM) was used to label mouse platelets as previously published by Moog et al. Whole blood was then reconstituted with the PRP containing the fluorescent platelets and 5 U/ml apyrase, before perfusion in the flow chamber. Under such conditions, mouse platelets were well labeled but the residual amount of free DIOC6 in whole blood was able to slightly label the endothelial cell monolayers during the blood perfusion of 3 min as it may be seen on figure 1B. This parasite fluorescence caused a weak background of ~7% which has been deduced from the percentage of the surface coverage measured in mouse platelet experiments.

**Flow chamber and in vitro flow studies**

Platelet interaction with endothelial cells was studied at shear rate of 100 s⁻¹ using a flow chamber purchased from Immunetics (Cambridge, MA) described elsewhere. It was designed to allow stabilized laminar flow between 0.1 and 2 dyn/cm². Whole blood was perfused through the chamber either on a monolayer of endothelial cells or immobilized peptide using withdrawal syringe pump (Harvard Apparatus, Boston, MA). Monolayers of
endothelial cells were obtained after 1 day of culture of 5 x 10^5 cells in Lab-TeK 1 chamber slides (Poly Labo, Strasbourg, France). Then, cells were stimulated or not for 18 h with 25 ng/ml TNF-α at 37 °C. The 4N1K and 4NGG-coated coverslips were prepared as follows: 3 ml of 50 or 100 µM of each peptide in DMEM medium were incubated in Lab-TeK 1 chamber slide 12 h at 4 °C, washing three times with PBS, saturated 2 h at room temperature with PBS/0.2% BSA and washed again with PBS before use. In some experiments 30 µg of rhICAM-1 in 3 ml of carbonate/bicarbonate buffer (pH 9.6) were added after the 4N1K peptide. Whole blood was perfused for 3 min. HBSS medium (Gibco Laboratories) was perfused to remove blood cells and non firmly adherent platelets before quantification.

**Platelet adhesion quantification**

The flow chamber, mounted on an epifluorescence inverted microscope Axiovert 25 B/W (Carl Zeiss, Oberkochen, Germany) allowed direct visualisation in real time of the platelet adhesion process. The microscope being coupled to a numeric camera of high resolution Axiocam (Carl Zeiss) directly linked to a pentium 3 PC computer (SCS, Antony, France) equiped with the acquisition software Axiovision (Carl Zeiss), ten random fields were recorded/coverlslip. Each image was subjected to computer-assisted analysis with the NIH-image 1.62/fat software (National Institutes of Health, Bethesda, MD). This program was used to calculate the percentage of the area covered by adhering platelets in a define area (surface coverage) after background substraction, setting the threshold value and binarization of each image. This technique implicates the use of an average value for adhesion of all platelets because it cannot discriminate between individual bound platelets and clumpings of platelets. Nevertheless, mainly individual platelets or very small aggregates are visible (Fig. 1A). Each experiment has been done at least three times.

**Flow cytometry analysis**

Endothelial cells (2 x 10^6 cells/ml) were incubated at room temperature in 200 µl DMEM medium containing the blocking mAb. Then, the cells were stained with FITC-conjugated Rabbit Anti-Mouse (RAM-FITC) immunoglobulins (Dako, Glostrup, Denmark) for 30 min at room temperature in the dark. Between each steps, cells were washed twice with DMEM medium. Controls included cells incubated with anti-CD20 (negative control) and anti CD47 (positive control) mAbs plus RAM-FITC. After a wash, cells were analyzed on a flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA).
Statistics

For overall comparison between groups nonparametric Kruskal-Wallis ANOVA was performed. For detection of differences between groups, Wilcoxon testing was used. P<0.05 was considered significant. Data are reported as mean ± S.E.M. (standard error of the mean).
RESULTS

*Platelet CD47 is involved in the adhesion of resting platelets to the inflammatory vascular endothelium*

To investigate a role for CD47 in platelet/endothelial cell interactions, we used an *in vitro* flow chamber assay that permits study of the dynamic aspects of platelet adhesion without altering endothelial cell integrity. When human platelets were allowed to flow in whole blood at $100 \text{s}^{-1}$ on a monolayer of resting vascular endothelial EA cells, as expected, no significant platelet adhesion was detected (Fig. 1A). When endothelial EA cells were pretreated with TNF-α, platelets did adhere ($p<0.001$). In such experimental conditions, the surface covered by platelets adhering to the endothelial monolayer varies between 8 and 12%. When the blocking anti-CD47 mAb "B6H12" was added to human platelets at a saturating concentration before flowing, platelets did not adhere on resting endothelial cells. They significantly adhered ($p<0.001$) on TNF-α-treated cells but a decrease of ~45% in platelet attachment ($p<0.01$) was observed (Fig. 1A). The addition of the non-relevant anti-CD20 mAb had no effect. Similar results were obtained with primary venous endothelial HUVEC cells (not shown). However, cells from the transformed vascular endothelial EA line have been chosen to perform the study since platelet adhesion on stimulated HUVEC cells resulted in large aggregates difficult to quantify. Since both platelets and endothelial cells express CD47, we also studied the role of endothelial CD47 in this process. The pretreatment of the endothelial cells with the blocking anti-CD47 mAb "B6H12" did not modify the adhesion of platelets (not shown) indicating that endothelial CD47 was not involved. Thus, these results disclose a role for platelet CD47 in the adhesion of platelets on inflamed vascular endothelium (IVE) at low shear rate.
Figure 1

A: Human

resting platelets
EA + TNF-α

resting platelets
EA + TNF-α + anti-CD47 mAb

resting endothelial cells

TNF-α-stimulated endothelial cells

B: Mouse

wild type platelets
EA + TNF-α

CD47-/- platelets
EA + TNF-α

resting endothelial cells

TNF-α-stimulated endothelial cells
Therefore, it was of interest to compare the adhesion of platelets from CD47-/- and C57BL/6 mice (Fig. 1B). While human platelet were labeled with calcein, mouse platelets were labeled with DIOC6 before flowing on the vascular endothelial cell monolayer stimulated or not with TNF-α (see Materials and Methods). Under such experimental conditions, very few mouse platelets arrested on resting endothelial cells when platelets from wild type and CD47-/- mice significantly attached (p<0.001) on TNF-α-stimulated cells. Nevertheless, although platelet concentration was similar in wild and CD47-/- mice, platelets from CD47-/- mice adhered significantly less (p<0.05) than platelets from wild type mice. A decrease of ~40% in platelet attachment was observed with platelets from CD47 deficient mice (Fig. 1B). Consequently, as observed with human platelets, these results demonstrate the involvement of platelet CD47 in the adhesion of resting platelets on IVE at low shear rate.

**Platelet CD47 binds the CBD domain of endothelial TSP-1**

To study the CD47 pathway, we first looked for the endothelial counterreceptors of platelet CD47. Thus far, thrombospondin-1 (TSP-1) and Signal Regulatory protein α (SIRPα) have been described to link CD47. As vascular endothelial cells display these two ligands for CD47 19,20, TNF-α-activated endothelial monolayers were treated or not with known blocking mAbs directed against TSP-1 and SIRPα. Figure 2 shows that the treatment of vascular endothelial cells with the blocking anti-TSP-1 mAb "C6.7", directed against the cell binding domain (CBD) of the molecule, inhibited the arrest of human platelets of ~50%. In contrast, the anti-TSP-1 mAb "A2.A", directed against the proteoglycan heparan sulfate region of TSP-1, had no effect. When the mAb "SE5A5" known to block the binding of SIRPα (SIRPα1 and SIRPα2) to CD47 was added, no effect on platelet arrest to IVE at low shear rate was observed (Fig. 2). Consequently, the CBD domain of the endothelial TSP-1 is a ligand of platelet CD47 in platelet/IVE interactions under flow conditions, whereas we found no evidence for a role of SIRPα in those events.
To check if platelet CD47 was able to directly link the CBD domain of TSP-1, we measured the arrest of human platelets, treated or not with the blocking anti-CD47 mAb "B6H12", on immobilized peptide 4N1K, derived from the CBD and on the immobilized control peptide 4NGG (Fig. 3). When resting human platelets were allowed to flow on 50 and 100µM-coated 4N1K or 4NGG peptides, platelets arrested and adhered at the two concentrations on 4N1K peptide (respectively ~10 and ~12 % of the surface covered) but no arrest was observed on the 4NGG control peptide. When platelets were preincubated with the blocking anti-CD47 mAb "B6H12" they no longer arrested on the 4N1K-coated surfaces. As the sequence of the CBD of human and mouse TSP-1 are identical, we compared, in the same experimental conditions, the arrest of platelets from wild and CD47/- mice on 100µM-coated peptides. Platelets from wild C57BL/6 mice adhered on 100µM-coated 4N1K peptide
(surface coverage ~13-15%) while they did not adhere on 100µM-coated 4NGG peptide (surface coverage ~1-2%). By contrast, platelets from CD47-/- mice arrested neither on the 4NGG nor on the 4N1K imobilized peptides (surface coverage ~1-2%) (not shown). Thus, platelet CD47 is able to directly link the CBD domain of TSP-1.

Figure 3

Soluble 4N1K peptide triggers platelet arrest on inflamed endothelium

In many cell types and in platelets, CD47 has been described to be physically linked to β1 3 and β3 2,16 integrins and, after its ligation with the CBD region of TSP-1, to stimulate their "activation" to a higher affinity/avidity state. To investigate if this happened during the adhesion of resting platelets on inflammatory endothelium, we incubated human platelets in PRP with the 4N1K or 4NGG peptides in solution before flowing. When platelets were preincubated with the peptide 4NGG, no modification in platelet arrest on TNF-α-stimulated EA cells was observed compared to the “basal arrest” (Fig. 4). Yet, when platelets were preincubated with the peptide 4N1K, an increase of ~40-50% in platelet adhesion was observed. This increase was completely abolished when platelets were first incubated in the
presence of the anti CD47 mAb "B6H12" (Fig. 4). Similar results, were obtained with primary endothelial HUVEC cells (not shown).

**Figure 4**

Furthermore, when platelets from C57BL/6 mice were incubated with the 4N1K peptide before flowing, an increase of ~45% in platelet arrest on the TNF-α-stimulated endothelial cell monolayers was observed compared to the “basal arrest”. But, no modification was seen when platelets were preincubated with the 4NGG peptide. With platelets from CD47−/− mice, no difference in platelet arrest was observed between resting or 4N1K or 4NGG stimulated platelets (not shown). Therefore, these results disclose an
activating effect of CD47 on platelet integrins in the interactions of platelets to the IVE at low shear rate.

**CD47 ligation activates the platelet αIIbβ3 integrin**

So far, CD47 was described to be linked and to activate three distinct integrins on nucleated cells or on platelets: αIIbβ3, α2β1 and αvβ3. Since these 3 integrins are expressed on platelet surface, we investigated whether they could be involved in platelet adhesion on IVE. For this purpose, platelets were first activated with the 4N1K peptide in solution and secondly, incubated in the presence of known blocking mAbs directed against each of these integrins. The addition of the anti-α2β1 mAb had no effect. Although a slight decrease (~18%) in platelet arrest was observed with the anti-αvβ3 mAb, a significant (p<0.001) decrease (~47%) was observed only with the anti-αIIbβ3 mAb (Fig. 5). Then, we found evidence that platelet CD47 ligation with a peptide derived from the CBD of TSP-1 induces the "activation" of the platelet αIIbβ3 integrin to a higher affinity/avidity state which allows its interaction with endothelial receptors.
**Endothelial ICAM-1 and αvβ3 integrin are involved in the CD47-dependent pathway**

As a study performed in static conditions reported that platelet αIIbβ3 interactions with endothelial ICAM-1, αvβ3 and GPIbα was involved in the adhesion of thrombin-activated human platelets to endothelial cells \(^ {21}\), we first investigated by flow cytometry, the expression of these three receptors on the vascular endothelial cells stimulated or not with 25 ng/ml TNF-α for 18h. In agreement with Mutin et al. \(^ {27}\) we confirmed that EA cells constitutively express ICAM-1 and αvβ3 and that upon TNF-α stimulation, the expression of αvβ3 was not modified when the expression of ICAM-1 was increased. But, we did not detect GPIbα on resting or TNF-α-stimulated EA cells (not shown).
Therefore, we first incubated TNF-α-stimulated cells with or without blocking mAbs directed against ICAM-1, the integrin αvβ3 or both. Next, the arrest of 4N1K-activated human platelets in whole blood was evaluated under flow (Fig. 6). Platelet arrest was significantly diminished (p<0.001) in the presence of the anti-ICAM-1 (decrease of ~50%), the anti-αvβ3 (decrease of ~25%) or both mAbs (decrease of ~68%). Nevertheless, it is noteworthy that we never could observe a total inhibition of platelet adhesion under flow. Thus, at least, these two endothelial receptors are implicated in platelet/IVE interaction at low shear rate.

Figure 6
These results are strengthened by the fact that human resting platelets significantly more adhered to 4N1K + ICAM-1-coated peptides compared to 4N1K coated alone (surface coverage respectively ~15% and ~7%) and did not adhere to ICAM-1 alone (Fig. 7). Furthermore, this increase in platelet arrest being inhibited in the presence of the blocking mAb P2 directed against the integrin αIIbβ3 (Fig. 7), this confirmed the involvement of this platelet integrin during resting platelet/IVE interactions under flow.

**Figure 7**

![Graph showing surface coverage percentage for different conditions: ICAM-1, 4N1K, 4N1K+ICAM-1, 4N1K+ICAM-1 + anti-αIIbβ3.]

**TSP-1/CD36 interaction would trigger the CD47-dependent pathway**

CD36 is a well known TSP-1 ligand which is spacially associated with the integrin αIIbβ3 on the surface of resting platelets. As the incubation of platelets with a
blocking mAb directed against CD36 resulted in a decrease in platelet arrest of ~55% (Fig. 8), platelet CD36 is involved in our phenomenon. TSP-1/CD36 interaction has been demonstrated to be a two-step process involving conformational changes in TSP-1. Thus, our results suggest that TSP-1/CD36 interaction could trigger conformational changes in endothelial TSP-1 leading to the exposure of its CBD domain, allowing the binding with platelet CD47. This hypothesis is strengthened by the fact that the incubation of anti-CD36-treated platelets with a blocking anti-CD47 mAb did not further decrease (~58%) platelet arrest on IVE (Fig.8).

As mentioned above, other endothelial receptors are involved. Furthermore, as no complete inhibition in platelet attachment to IVE was reached by blocking or deleting CD47, other platelet receptors would be implicated too. Interactions involving endothelial vWF and platelet GPIbα were demonstrated to happen in mice at low shear rate (80-100 s⁻¹). Endothelial PECAM-1 was described to contribute to platelet adhesion at a site of injured but not denuded endothelium. As this receptor performs its adhesive functions via PECAM-1/PECAM-1 homophilic interactions and as resting platelets express PECAM-1, this pathway could be involved too. However, no inhibition in platelet arrest was observed when platelets were incubated with a blocking anti-PECAM-1 mAb when a decrease of ~68% was observed when they were incubated with a blocking anti-GPIbα mAb (not shown). Thus, other pathway(s) cooperate with the CD47-dependent pathway in the adhesion of resting platelets to IVE in dynamic conditions.
Figure 8

Surface Coverage (%)

- Whole Blood
- + anti-CD36
- + anti-CD4 7
- + 2 mAbs

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DISCUSSION

In thrombus formation associated with hemostasis or thrombotic disease, blood platelets first undergo a rapid transition from a circulating state to an adherent state, followed by activation and aggregation. Under flow conditions in the bloodstream, this process potentially involves platelet-platelet, platelet-endothelium and platelet-subendothelial matrix interactions. Specific adhesion receptors on platelets mediate these interactions, by engaging counterreceptors on endothelial cells, or non-cellular ligands in the plasma or matrix. While adhesion receptors involved in platelet-matrix interactions have been studied extensively, those involved in platelet-endothelium interactions are incompletely characterized and have been mainly studied in static conditions with washed platelets. Here, we identified a CD47-dependent pathway acting in cooperation with other pathways involving platelet GPIbα, CD36 but not platelet PECAM-1. Using a flow chamber, we report that at low shear rate, CD47 on human platelets in whole blood significantly contributes to platelet adhesion on TNF-α-stimulated endothelial cells. CD47 binds to the CBD domain of the endothelial TSP-1; it thus induces the activation of the platelet αIIbβ3 integrin which, in turn, becomes able to link the endothelial receptors ICAM-1 and αvβ3. The main points of this study were confirmed with platelets from wild type and CD47-/- mice.

Using blocking mAbs directed against the two known ligands of CD47: TSP-1 and SIRPα, we observed no inhibition in platelet arrest in the presence of the anti-SIRPα or the mAb directed against the proteoglycan heparan region of TSP-1 when an inhibition of ~50% was obtained in the presence of a mAb directed against the CBD domain of TSP-1. Under flow conditions, the ligand of the endothelial TSP-1 on platelets has never been clearly established. CD47 was initially reported to have no functional role in platelets 35. Since then, under static conditions, platelet CD47 has been described to be involved in platelet activation and aggregation 3 and platelet spreading on fibrinogen-coated surfaces 2. In all these phenomena, the CBD region of TSP-1 has been shown to be the ligand for CD47, except for Tulasne et al. 36 who recently demonstrated that the c-terminal peptide of TSP-1 induced platelet aggregation through the FcR-γ chain signaling pathway. A previous work indicated that immobilized TSP-1 could induce platelet arrest and that shear rate influence this adhesion 37. Our study confirms and completes this observation since we showed that
platelets adhere on the immobilized 4N1K peptide (a peptide derived from the CBD region of TSP-1), and this adhesion is inhibited in the presence of a neutralizing mAb directed against CD47, indicating a direct association of platelet CD47 with the CBD region of TSP-1 at low shear rate. As a deep decrease in platelet arrest was observed on immobilized 4N1K at high shear rate (1200s\(^{-1}\)) (not shown), it seems that this interaction can be established only at low shear rate and/or is not sufficient at high shear rate.

In platelets, CD47 has been described to be physically linked to \(\beta_1\) \(^3\) and \(\beta_3\) \(^2,16\) integrins and to form a signal transducing complex allowing their activation to a higher affinity/avidity state after its ligation with the CBD region of TSP-1 in static conditions. For instance, incubation of washed platelets in the presence of the 4N1K peptide was shown to stimulate platelet spreading on fibrinogen-coated surfaces and to induce their aggregation via activation of the integrin \(\alpha_{IIb}\beta_3\) \(^2\) and to synergise with collagen in \(\alpha_2\beta_1\)-mediated platelet activation \(^3\). Although 4N1K alone failed to induce platelet aggregation in PRP \(^38\), we observed that the incubation of platelets in the presence of the 4N1K peptide in PRP before blood reconstitution, resulted in a significant enhancement of platelet arrest on the TNF-\(\alpha\)-stimulated endothelial cells. Since this increase was inhibited with a blocking anti CD47 mAb in our experimental conditions, it likely indicates that platelet CD47 is able to positively regulate the integrin activity not only in static but in dynamic conditions too. CD47 was described to regulate the activity of \(\alpha_{IIb}\beta_3\) \(^2\) and \(\alpha_2\beta_1\) \(^3\) integrins in platelets but also of the integrin \(\alpha_v\beta_3\) in fibroblasts \(^39\) and in melanoma cells \(^17\). Although these three integrins are expressed on platelets, only the integrin \(\alpha_{IIb}\beta_3\) appeared to be activated following the incubation with the 4N1K peptide in our experimental model. As this work was performed with whole blood, we cannot exclude a possible activation of the integrin \(\alpha_{IIb}\beta_3\) by factors like thrombin, ADP, epinephrine and thromboxane A2. However, their roles seem unlikely since the experiments were done with heparinized blood in the presence of apyrase (an ADP scavenger) and since results were not modified in the presence of 2U/ml of hirudin (a thrombin inhibitor, not shown). Moreover, such an involvement of the integrin \(\alpha_{IIb}\beta_3\) has already been described in the interaction of ADP activated platelets on non inflammatory endothelium under flow conditions \(^40\) and thrombin activated platelets under static conditions \(^25\).
Using neutralizing mAbs, we next identified ICAM-1 and αvβ3 integrin as endothelial receptors involved in resting platelet/IVE interactions, ICAM-1 being the predominant receptor. The anti-ICAM-1 mAb B-H17 used in our study was previously described to inhibit the ligation of ICAM-1 to the αLβ2 integrin (LFA-1, CD11a-CD18) and, the binding site involved in this interaction was also implicated in the binding of ICAM-1 to the fibrinogen. As the αIIbβ3 integrin serves as the main receptor for fibrinogen, this strongly suggests that resting platelet bind to TNF-α-stimulated endothelial cells by an αIIbβ3-dependent bridging mechanism: the fibrinogen making the bridge between endothelial ICAM-1/αvβ3 and platelet αIIbβ3 integrin. Likewise, as demonstrated by flow cytometry in the adherence of thrombin activated platelets to HUVEC, the participation of fibronectin, vitronectin and vWF as other bridges is probable. No complete inhibition of platelet adhesion was observed in the presence of the two mAbs directed against ICAM-1 and αvβ3. In the same way, blocking or deleting the CD47 pathway did not induce a complete inhibition of platelet adhesion on IVE since a residual arrest of ~ 50% stayed, indicating that other endothelial and platelet receptors would act in synergy. In vivo, rolling is indispensable before firm adhesion, for normal platelet function as it serves to decelerate cell movement relative to flowing blood, allowing receptors with slower bond kinetics (ie, integrins) to engage adhesive ligands. Using intravital microscopy, endothelial P- and E-selectin, as well as vWF, have been demonstrated to mediate platelet rolling. Considering the short-lasted expression of the selectin in vitro (few hours), their participation in our experimental conditions seems unlikely. But, the pathway involving the vWF secreted on the luminal face of endothelial cells and the platelet GPIbα must be implicated since the addition of a blocking mAb directed against platelet GPIbα decreases platelet adhesion of ~65-70%. In accordance with Perrault et al., we detected no expression of GPIbα on resting or TNF-α-stimulated EA cells excluding its participation in our phenomenon. An in vivo study presented evidence that endothelial PECAM-1 contributed to platelet adhesion at a site of injured but not denuded endothelium. However, the addition of a blocking mAb directed against either endothelial or platelet PECAM-1 at a saturating concentration did not modified platelet arrest in our model. On the other hand, the presence of a blocking anti-CD36 mAb in the PRP significantly decreased platelet arrest, strongly suggesting that the endothelial TSP-1 has, at least, two platelet receptors: CD36 and CD47.
Normally, endothelial cells act as nonthrombogenic surface. But, inflammatory stimuli (TNF-α, IL-1β…) induce phenotypical and conformational changes in the surface receptors of endothelial cells, allowing resting platelets to adhere. Using flow cytometry, we confirmed that EA endothelial cells express TSP-1, αvβ3 and ICAM-1. Eighteen hours of TNF-α-stimulation induced an increase in ICAM-1 expression (not shown). Thus, considering litterature and our data, we can propose the following model. After endothelial activation under low shear conditions, the interaction between platelet GPIbα and the vWF expressed on endothelial cells initiates platelet rolling 10, allowing platelet CD36 and endothelial TSP-1 ligation. CD36/TSP-1 interaction is a two-step process 30. The sequence 139-155 region of CD36 binds first to a sequence localized in the type 1 repeated unit domain of TSP-1, triggering a conformational change in TSP-1 to reveal a second site in the C-terminal domain of TSP-1, which links the 93-110 region of CD36 with high affinity. As the CBD domain of TSP-1 is localized in its carboxyl-terminal domain 26, we hypothesize that the TSP-1/CD36 interaction induces the exposition of the CBD domain, allowing its ligation with the platelet CD47 which results in platelet αIIbβ3 integrin activation. The αIIbβ3 integrin in its active form can then links endothelial αvβ3 and overexpressed ICAM-1 through fibrinogen bridges as described above, allowing firm adhesion of platelets. TSP-1 was also demonstrated to be non-adhesive in a Ca(2+)-depleted conformation, when supporting platelet adhesion in a Ca(2+)-repleted conformation 37. Using a mAb which differentially labels these two conformations of TSP-1 45, we confirmed with flow cytometry that in our experimental conditions, EA cells express TSP-1 in its Ca(2+)-repleted conformation, but no change was observed upon TNF-α stimulation (not shown).

In conclusion, we disclosed a role for CD47 in the adhesion process of resting platelets to inflamed vascular endothelium at low shear rate. Such platelet/endothelium interaction is thought to play a causal role during atherogenesis 46,47 and, to represent the initial event leading to remodeling and reocclusion of the vasculature 6,48. It has also been demonstrated to participate to several diseases of the central nervous system (autoimmune inflammatory diseases, stroke…) by recruiting leukocytes 49. Thus, CD47 antagonists would be of significant clinical importance.
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FIGURE LEGENDS

Figure 1: Involvement of platelet CD47 in the adhesion of resting platelets on the inflammatory vascular endothelium under flow conditions

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated or not with 25 ng/ml rhTNF-α for 18 h. Then, the slides were placed in a parallel plate flow chamber that produces a linear variable shear rate.

A: Human platelets were labeled with calcein in the PRP and then, incubated or not, with 40 µg/ml anti-CD47 mAb "B6H12" for 10 min at 37°C. Whole blood was reconstituted and perfused through the chamber at 37°C. Single-frame images were obtained after perfusion for 3 min at shear rate of 100s⁻¹ on EA cells treated or not with TNF-α. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001. These experiments are representative of at least 8 experiments performed with blood from different donors. For each single-frame image shown in the panels the magnification of the objective was 10 x.

B: Mouse platelets from 10 wild-type or 10 CD47-/- C57BL/6 mice were labeled with DIOC6 in the PRP (see "materials and methods"). Whole blood was reconstituted and perfused through the chamber at 37°C. Single-frame images were obtained after perfusion for 3 min at shear rate of 100s⁻¹ on EA cells treated or not with TNF-α. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001. These experiments are representative of 4 different experiments. For each single-frame image shown in the panels the magnification of the objective was 10 x.

Figure 2: Arrest of flowing platelets in whole blood on TNF-α-treated vascular endothelial cells incubated with blocking mAbs directed against TSP-1 or SIRPα

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated with 25 ng/ml rh TNF-α for 18 h. Then, 40 µg/ml of anti-TSP1 "C6.7" and "A2.5" or anti-SIRPα "SE5A5" mAbs were added or not (control) to these cells for 10 min at 37°C. They were washed with PBS before to be placed in the flow chamber. After platelet labeling in PRP with calcein, whole blood was reconstituted and perfused through the chamber at 37°C at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001.
This experiment is representative of 3 experiments performed with blood from different donors.

Figure 3: Arrest of flowing platelets in whole blood on immobilized 4N1K and 4NGG peptides

Permanox Lab-Tek 1 chamber slides were coated with 50 or 100µM of 4N1K (a CD47 agonist from the CBD of TSP1) or 4NGG (control) peptides and placed in the flow chamber. Platelets were first labeled with calcein in PRP and then, incubated or not with 40 µg/ml anti-CD47 mAb "B6H12" for 10 min at 37°C. Whole blood was reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance : *** P<0.001. This experiment is representative of 3 experiments performed with blood from different donors.

Figure 4: Arrest of flowing platelets incubated with or without the 4N1K peptide in solution on TNFα-treated endothelial cells

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated with 25 ng/ml rh TNF-α for 18 h. After platelet labeling in PRP with calcein, platelets were incubated 15 min at 37°C with or without 100 µM 4N1K or 4NGG peptides in solution. In some experiments platelets were first incubated 10 min at 37°C with 40 µg/ml of anti CD47 mAb "B6H12" before the stimulation with the 4N1K peptide. Whole blood was then reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance : *** P<0.001 ; ** P<0.01. This experiment is representative of 3 experiments performed with blood from different donors.

Figure 5: Arrest of 4N1K activated platelets incubated with blocking mAbs directed against the integrins αIIbβ3, α2β1 or αvβ3 on TNF-α-treated endothelial cells

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated with 25 ng/ml rh TNF-α for 18 h. After platelet labeling in PRP with calcein, platelets were incubated 15 min at 37°C with or without 100 µM 4N1K peptide in solution followed by 10 min incubation at 37°C with 40 µg/ml of either the anti-αIIbβ3 mAb "P2" or the anti-α2β1 mAb "BHA2.1" or the anti-αvβ3 mAb "LM609". Whole blood was then
reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001. This experiment is representative of 3 experiments performed with blood from different donors.

Figure 6: **Arrest of 4N1K activated platelets on TNF-α-treated endothelial cells incubated with blocking mAbs directed against ICAM-1 and/or the αvβ3 integrin**

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated with 25 ng/ml rh TNF-α for 18 h. Then, 40 µg/ml of anti-ICAM-1 "B-H17" or anti αvβ3 "LM609" or both mAbs were added to these cells for 10 min at 37°C. They were washed with PBS before to be placed in the flow chamber. After platelet labeling in PRP with calcein, platelets were incubated 15 min at 37°C with or without 100 µM 4N1K peptide in solution. Whole blood was then reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001. This experiment is representative of 3 experiments performed with blood from different donors.

Figure 7: **Arrest of flowing platelets incubated with or without a blocking anti-αIIbβ3 mAb on immobilized peptides 4N1K ± ICAM-1**

Permanox Lab-Tek 1 chamber slides were coated with 100 µM of 4N1K ± 30 µg of rhICAM-1 peptides (see "materials and methods") and placed in the flow chamber. Platelets were first labeled with calcein in PRP and then, incubated or not with 40 µg/ml anti-αIIbβ3 mAb "P2" for 10 min at 37°C. Whole blood was reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/ coverslip. Statistical significance: *** P<0.001. This experiment is representative of 3 experiments performed with blood from different donors.

Figure 8: **Arrest of flowing platelets incubated with or without a blocking anti-CD36, an anti-CD47 or the 2 mAbs successively on TNF-α-treated endothelial cells**

EA endothelial cells were grown onto permanox Lab-Tek 1 chamber slides and stimulated with 25 ng/ml rh TNF-α for 18 h. Platelets were first labeled with calcein in PRP and then, incubated or not with 40 µg/ml anti-CD36 mAb "FA6.152" or anti-CD47 mAb
"B6H12" for 10 min at 37°C or the two mAbs successively. Whole blood was reconstituted and perfused through the chamber at 37°C, at 100s⁻¹ for 3 min. Platelet adhesion, expressed as percent surface covered with platelets, is the average ± S.E.M. of 10 random fields/cover slip. Statistical significance: *** P<0.001. This experiment is representative of 3 experiments performed with blood from different donors.
Involvement of a CD47-dependent pathway in platelet adhesion on inflamed vascular endothelium under flow

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