Characterization of a Novel Function-Blocking Antibody Targeted Against the Platelet P2Y<sub>1</sub> Receptor


Objective—Platelet hyperactivity is associated with vascular disease and contributes to the genesis of thrombotic disorders. ADP plays an important role in platelet activation and activates platelets through 2 G-protein–coupled receptors, the Gq-coupled P2Y<sub>1</sub> receptor (P2Y<sub>1</sub>R), and the Gi-coupled P2Y<sub>12</sub> receptor. Although the involvement of the P2Y<sub>1</sub>R in thrombogenesis is well established, there are no antagonists that are currently available for clinical use.

Approach and Results—Our goal is to determine whether a novel antibody targeting the ligand-binding domain, ie, second extracellular loop (EL2) of the P2Y<sub>1</sub>R (EL2Ab) could inhibit platelet function and protect against thrombogenesis. Our results revealed that the EL2Ab does indeed inhibit ADP-induced platelet aggregation, in a dose-dependent manner. Furthermore, EL2Ab was found to inhibit integrin GPIIb-IIIa activation, dense and α granule secretion, and phosphatidylserine exposure. These inhibitory effects translated into protection against thrombus formation, as evident by a prolonged time for occlusion in a FeCl<sub>3</sub>-induced thrombosis model, but this was accompanied by a prolonged tail bleeding time. We also observed a dose-dependent displacement of the radiolabeled P2Y<sub>1</sub>R antagonist [H]MRS2500 from its ligand-binding site by EL2Ab.

Conclusions—Collectively, our findings demonstrate that EL2Ab binds to and exhibits P2Y<sub>1</sub>R-dependent function-blocking activity in the context of platelets. These results add further evidence for a role of the P2Y<sub>1</sub>R in thrombosis and validate the concept that targeting it is a relevant alternative or complement to current antiplatelet strategies. (Arterioscler Thromb Vasc Biol. 2015;35:637-644. DOI: 10.1161/ATVBAHA.114.304509.)

Key Words: antiplatelet agents ▪ antithrombotic agents ▪ blood platelets ▪ P2Y<sub>1</sub> receptor antagonists ▪ platelet inhibitors

ADP released from platelets, red blood cells, and damaged blood vessels is a key activator of platelets and plays an important role in generation of arterial thrombi at the site of vascular injury. Two G-protein–coupled receptors, P2Y<sub>1</sub> and P2Y<sub>12</sub>, are required for full ADP-induced platelet aggregation, but each of these receptors plays a different role in this process. P2Y<sub>1</sub> receptor (P2Y<sub>1</sub>R) triggers a rapid and transient intracellular calcium increase that causes platelet shape change at the P2Y<sub>1</sub>R. It is noteworthy that pharmacological studies and studies with P2Y<sub>1</sub>R-deficient mice indicate that these receptors could also be relevant targets for new antiplatelet compounds.

About the P2Y<sub>1</sub>R, studies with P2Y<sub>1</sub>R<sup>−/−</sup> mice indicated defective aggregation in response to ADP and low concentrations of collagen. These animals displayed resistance to systemic thromboembolism induced by infusion of either a mixture of collagen and adrenaline or tissue factor, pointing to the essential role of this receptor in thrombin-dependent or thrombin-independent model of thrombosis. Moreover, in a model of localized arterial thrombosis of mesenteric arterioles triggered by ferric chloride injury, P2Y<sub>1</sub>R<sup>−/−</sup> mice also displayed reduced and delayed thrombus formation when compared with the wild type. In this model, the extent of inhibition was found to be equivalent to that of clopidogrel-treated animals at the maximal effective dose. In addition, the combination of P2Y<sub>1</sub>R deficiency and clopidogrel treatment was found to be more efficient than each alone, opening the possibility that a combination of P2 receptor antagonists could improve anti-thrombotic strategies. Altogether, these results suggested the P2Y<sub>1</sub>R to be a potential target for new antiplatelet compounds.
To this end, the present studies characterized the biological activity of a novel antibody targeted against the ligand-binding domain of the platelet P2Y<sub>1</sub>R (ie, second extracellular loop [EL2]) abbreviated as EL2Ab in the context of platelet function. The EL2Ab was found to recognize the P2Y<sub>1</sub>R protein specifically and to inhibit aggregation of human and mouse platelets induced by ADP, in a dose-dependent manner, whereas it produced no effects on aggregation mediated by the thromboxane receptor (TPR), or in response to thrombin and collagen stimulation. Moreover, the EL2Ab also inhibited GP IIb-IIIa activation, platelet dense and α granule secretion, and phosphatidylserine exposure. Radioligand binding studies revealed that the mechanism by which EL2Ab exerts these effects involves interaction/antagonism of the P2Y<sub>1</sub>R. Importantly, our data provide evidence that EL2Ab does not interact with the P2Y<sub>12</sub>R. Considering the biological function of the P2Y<sub>1</sub>R, it could be hypothesized that an antibody raised against the receptor’s ligand-binding domain (ie, EL2) should in turn prevent thrombosis much in the same way as an antagonist. Indeed, in vivo analysis demonstrated that although EL2Ab prolongs the time for occlusion and protects against thrombogenesis, it does so accompanied by impairing hemostasis and prolonging tail bleeding time.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**EL2Ab Recognizes the P2Y<sub>1</sub>R in Human and Mouse Platelets**

The purpose of these studies is to provide evidence that the EL2Ab, which was generated against the EL2 region of the P2Y<sub>1</sub>R, does indeed have the capacity to recognize the receptor protein, in both human and mouse platelet preparations. Thus, in our initial characterization, we used Western blotting and flow cytometry analysis of the P2Y<sub>1</sub>R using the EL2Ab. Consequently, our Western blot data revealed that when the EL2Ab was used as the primary antibody, it did in fact recognize the P2Y<sub>1</sub>R protein, with a band observed at ≈47 kDa, in both human (Figure 1A) and mouse platelets (Figure 1B). On the contrary, when the EL2Ab was first preabsorbed with its cognate peptide (EL2; 100 μmol/L) no band was observed, indicating that it was no longer capable of recognizing the P2Y<sub>1</sub>R protein, with a band observed at 47 kDa, in both human (Figure 1A) and mouse platelets (Figure 1B). On the contrary, when the EL2Ab was first preabsorbed with its cognate peptide (EL2; 100 μmol/L) no band was observed, indicating that it was no longer capable of recognizing the P2Y<sub>1</sub>R protein, with a band observed at 47 kDa, in both human (Figure 1A) and mouse platelets (Figure 1B).

**Figure 1.** Second extracellular loop (EL2) of the P2Y<sub>1</sub> receptor (P2Y<sub>1</sub>R; EL2Ab) recognizes P2Y<sub>1</sub>R in platelets. EL2Ab was generated as discussed in the Materials and Methods in the online-only Data Supplement and shown to recognize both human and murine platelets by Western blotting (A and B), as well as flow cytometry (C and D). The P2Y<sub>1</sub>R<sup>−/−</sup> and wild-type platelets were subjected to Western blot (E) and flow cytometry (F) using the EL2Ab.
was generated against the human EL2 domain, exhibits inter-
species reactivity, at least toward the mouse receptor, and
under denatured protein conditions. In separate positive con-
trol studies, Western blot was conducted using a commercial
anti-human P2Y, R antibody. The results revealed a band cor-
responding to the P2Y, R protein at 47 kDa, in both human
and mouse platelets (Figure 1A and 1B), similar to what was
observed with EL2Ab under similar experimental conditions.
In the next set of experiments, we investigated the capacity
of the EL2Ab to recognize the native P2Y, R protein by using
flow cytometry analysis. It was found that incubating human
and mouse platelets with the EL2Ab resulted in a significa-
cnt rightward shift in the mean fluorescence intensity, when
compared with the normal rabbit IgG control (Figure 1C and
1D). Conversely, the preabsorbed EL2Ab did not produce a
significant rightward shift in the mean fluorescence inten-
sity (Figure 1C and 1D), ie, similar to what was found with
normal rabbit IgG. This finding supports the specificity of
the EL2Ab and the observed response. Moreover, separate posi-
tive control studies using a commercial anti-human P2Y, R
antibody also revealed a rightward shift in the mean fluo-
rescence intensity in human and mouse platelets (Figure 1C and
1D), which was comparable with what we observed with the
EL2Ab. In a separate set of control experiments, EL2Ab was
found to recognize the P2Y, R protein in the wild-type but not
in the P2Y, R−− platelets, further supporting the specificity of
the EL2Ab (Figure 1E). Moreover, flow cytometric analysis
revealed similar results (Figure 1F).

Taken together, these findings clearly establish that the
EL2Ab, which was raised against the human EL2 sequence,
exhibits interspecies reactivity, and that it has the capacity to
recognize the denatured and the native forms of the human,
as well as the mouse P2Y, receptor protein, at least for those
receptors that are known to be expressed on platelets.

Figure 2. Second extracellular loop (EL2) of the P2Y, receptor (EL2Ab) inhibits platelet aggregation in vitro and ex vivo. Platelets were
incubated with different concentrations of EL2Ab (3.125–12.5 ng) for 3 minutes and stimulated with either 5 μmol/L, ADP (A); 1 μmol/L,
U46619 (B); 0.1 U/mL thrombin (C); or 5 μg/mL collagen (D). Each experiment was repeated 3×, with blood obtained from 3 separate
donors. For the ex vivo studies, mice were injected with 12.5 ng of EL2Ab or IgG, and blood/platelets were collected 1 hour post injec-
tion. Platelets were stimulated with either 5 μmol/L, ADP (E) or 1 μmol/L, U46619 (F). Each experiment was repeated ≥3×, with blood
pooled from ≥3 separate groups of 8 mice.
EL2Ab Inhibits Platelet Aggregation of Human and Murine Platelets: In Vitro

Previous work has demonstrated that EL2 of P2Y1R contains an important ligand-binding pocket. Thus, we proposed that an antibody targeting this region (EL2Ab) would significantly block ADP-induced platelet aggregation. In our initial experiments, we showed that 6.25 ng of EL2Ab significantly inhibited human platelet aggregation induced by 5 μmol/L ADP when compared with normal rabbit IgG (Figure 2A). Furthermore, this inhibition was found to be dose dependent (Figure 2A). On the contrary, control experiments revealed that the EL2Ab even at 12.5 ng did not produce any detectable effects on platelet activation by the TPR agonist U46619 (1 μmol/L; Figure 2B), thrombin (0.1 U/mL; Figure 2C), or collagen (5 μg/mL; Figure 2D). The specificity of this effect was further demonstrated by the loss of EL2Ab ability to inhibit aggregation after preabsorption with 100 μmol/L of its cognate peptide (data not shown). Similar results were also obtained under in vitro conditions in mouse platelets (data not shown). Together, these findings indicate that EL2Ab inhibitory effects are selective to the P2Y1R, but not to the TPR.

EL2Ab Inhibits Platelet GPIIb-IIIa Activation

We next investigated whether the blockade of ADP-induced platelet aggregation by EL2Ab would be associated with a commensurate inhibition of GPIIb-IIIa activation. Indeed, our results showed that pretreating platelets with 12.5 ng of EL2Ab significantly reduced 5 μmol/L ADP-triggered PAC-1 (platelet activation complex-1) binding, indicating inhibition of GPIIb-IIIa activation (Figure 3A). On the contrary, 12.5 ng of EL2Ab had no detectable effects on 1 μmol/L U46619/TPR-induced GPIIb-IIIa activation (Figure 3B).

EL2Ab Inhibits Platelet α and Dense Granule Secretion

Platelet secretion is an important and early event in platelet activation that is known to be triggered by the agonist ADP. Thus, we sought to determine whether the EL2Ab would exert inhibitory effects on platelet secretion. It was found that 12.5 ng of EL2Ab significantly inhibited platelet α and dense granule secretion stimulated by 5 μmol/L ADP (Figure 3C and 3E, respectively), when compared with normal rabbit IgG (not shown). Conversely, no detectable effects on dense and α granule secretion were observed when platelets were activated by U46619 (1 μmol/L; Figure 3D and 3F, respectively). These data show that EL2Ab does indeed exert inhibitory effects on dense and α granule secretion.

EL2Ab Inhibits Platelet Phosphatidylserine Exposure

Exposure of platelet membrane phosphatidylserine regulates blood coagulation by modulating the production of thrombin. We examined whether the EL2Ab interaction with the P2Y1R would interfere with phosphatidylserine exposure. Indeed, our studies demonstrated that pretreatment of platelets with EL2Ab (12.5 ng) significantly inhibited 5 μmol/L ADP-induced

Figure 3. GPIIb-IIIa (PAC-1 [platelet activation complex-1] binding), P-selectin, dense granule secretion, and phosphatidyserine (PS) exposure (annexin V) were inhibited by EL2Ab, in human platelets. Washed platelets were incubated in the presence or absence of Second extracellular loop (EL2) of the P2Y1 receptor (EL2Ab; 12.5 ng) for 3 minutes and then stimulated with ADP (5 μmol/L) and U46619 (1 μmol/L) for 3 minutes. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 minutes at room temperature. A and B, Platelets were incubated with fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody, the fluorescent intensities were measured by flow cytometry, and the data were plotted as histogram. C and D, Platelets were incubated with FITC-conjugated anti-P-selectin antibody, the fluorescent intensities were measured by flow cytometry, and the data were plotted as histogram. G and H, Platelets were incubated with FITC-conjugated annexin V antibody, the fluorescent intensities were measured by flow cytometry, and the data were plotted as histogram. Platelets were incubated with EL2Ab (12.5 ng) for 3 minutes. 12.5 μL of luciferase luciferin was added and stimulated with either 5 μmol/L ADP (E) or 1 μmol/L U46619 (F) for 5 minutes. Release of ATP as a luminescence was measured by aggregometer. Each experiment was repeated ≥3×, with blood obtained from 3 separate donors.
phosphatidylserine exposure (Figure 3G), whereas it exerts no effect on that stimulated by 1 μmol/L U46619 (Figure 3H). These data provide evidence that the mechanism by which EL2Ab inhibits platelet function involves phosphatidylserine exposure.

**EL2Ab Competitively Displaces Radiolabeled [3H] MRS2500 From P2Y1 R Ligand-Binding Sites**

To define the molecular mechanism by which EL2Ab blocks P2Y1-R-mediated platelet activation, a radiolabeled ligand-binding assay was performed. Because this antibody was raised against second extracellular loop of P2Y1 receptor, which is part of the ligand-binding pocket, it would be expected to compete with other ligands for receptor binding effectively. Thus, platelets were incubated with the radiolabeled P2Y1 R antagonist [3H]MRS2500, and increasing concentration of EL2Ab were added (1–15 ng). We observed a dose-dependent displacement of [3H]MRS2500 from its P2Y1R ligand-binding sites by EL2Ab (Figure 4A); this effect was found to be reversed by preabsorption of EL2Ab with its cognate peptide (Figure 4A). Thus, the EL2Ab clearly binds to platelet P2Y1Rs in a specific manner, which is consistent with its antiplatelet effects.

**EL2Ab Does Not Interact With P2Y12 R**

In platelets, the agonist ADP is known to activate 2 separate G-protein-coupled receptors, namely the P2Y1 R, and the P2Y12 R (known to couple to Go). To confirm the selectivity of the EL2Ab for the P2Y1 R, and examine whether it can inhibit P2Y12-dependent activity, a cAMP assay was conducted using human platelets, as we described before.21–25 Our findings demonstrate that 5 μmol/L of ADP significantly lowered/inhibited 0.5 μmol/L forskolin-induced increases in cAMP levels (Figure 4B). Furthermore, pretreatment with 12.5 ng of the EL2Ab, which almost completely inhibited the aggregation response, did not produce any apparent effects on the ability of ADP (5 μmol/L) to lower cAMP levels that were elevated by 0.5 μmol/L of forskolin (Figure 4B), unlike 1 μmol/L of the P2Y12 antagonist AR-C 66096 (Figure 4B). Thus, these data provide evidence that the inhibitory effects the EL2Ab exerts on platelets are exclusively dependent on its interaction with the P2Y1 R.

**EL2Ab Inhibits Murine Platelet Aggregation: Ex Vivo**

Our initial experiments showed that EL2Ab inhibits ADP-induced platelet aggregation in human and in mouse platelets under in vitro experimental conditions. Thus, to examine a potential antithrombotic activity for EL2Ab better, and to guide the selection of doses for our in vivo studies, we decided to examine whether injections into live animals would also translate into inhibition of platelet aggregation. Indeed, it was found that 1 hour post tail-vein injections of 12.5 ng of EL2Ab administered intravenously into mice produced significant inhibition of ADP-stimulated aggregation (5 μmol/L; Figure 2E), whereas no effects were observed with the TPR agonist U46619 (1 μmol/L; Figure 2F). Hence, these data suggest that EL2Ab does have the capacity to inhibit platelet function even when injected into live animals.

**EL2Ab Inhibits Thrombogenesis and Hemostasis**

Because actin-dependent myosin contractility is thought to contribute to thrombus stability in the arterial system,26 we sought to assess the pharmacological relevance of the EL2Ab in vivo, by conducting a murine FeCl3 carotid artery injury thrombosis experiments. We found that the EL2Ab-treated animals (12.5 ng IV injections) had a prolonged time for occlusion (Figure 5A), when compared with normal rabbit IgG (Figure 5A). Furthermore, this prolongation was compatible with that observed with the experimental P2Y1 R antagonist MRS2500 (2 mg/kg; Figure 5A). We next investigated whether the EL2Ab would exert negative consequences on hemostasis by measuring the tail bleeding times. It was found that mice injected with 12.5 ng EL2Ab significantly prolonged tail bleeding times when compared with control animals (Figure 5B), when compared with IgG control (Figure 5B). Again, the P2Y1 R MRS2500 exerted similar effects on the tail bleeding time (Figure 5B). Taken together, the above data provide evidence that the EL2Ab not only exhibits antithrombotic activity but also increases the risk of bleeding.

**Discussion**

Whereas platelet activation is an integral part of hemostasis, vessel disease and vascular lesions are also major causative factors in arterial thrombosis. Thus, agents that interfere with the activation of platelets by their agonists have traditionally served as potential candidates for antithrombotic therapy.27,28
For example, drugs that target ADP-signaling pathways for pharmacological inhibition have been promoted as logical therapeutic approaches because of the presumed importance of secreted ADP in propagating the platelet aggregation response induced by numerous agonists. Of the 2 ADP-receptor signaling pathways in platelets, evidence has indicated that ADP-mediated P2Y₁₂ signaling seems to play a more prominent role in platelet activation than the P2Y₁ pathway. For the most part, support for this notion derives from the use of P2Y₁₂ antagonists which, unlike P2Y₁ antagonists, substantially inhibit aggregation to many platelet agonists, including thromboxane A₂, thrombin, and collagen. However, there is an accumulating evidence that this potent biological activity is the underlying cause for the significant bleeding phenotype associated with the use of P2Y₁₂ antagonists (eg, ticlopidine and clopidogrel; Plavix), which may limit their clinical utility. Thus, it is reasonable to suggest that a less-potent inhibitor (with a narrower spectrum of activity) may serve as a safer alternative. Consequently, antagonism of the P2Y₁ receptor, which was established using transgenic and pharmacological approaches as a mediator in thrombotic disease, has re-emerged as an alternative approach for targeting the ADP pathway and for managing such disease states. In fact, historically, the P2Y₁ receptor is the one thought to be responsible for the initiation of aggregation in response to ADP, but not the receptor that is targeted by clopidogrel (ie, the P2Y₁₂ receptor). It is noteworthy that despite an apparent success with the design of small-molecule P2Y₁ antagonists, unfortunately, each seemed to exhibit a certain handicapping deficiency (ie, limitations ranged from limited in vivo effectiveness, short biological half-life, toxicity, to poor potency, as well as oral bioavailability issues). One apparent reason for these failures is because these agents were empirically designed based on the structures of ADP or ATP, with little information about the actual P2Y₁ receptor binding domains. On the basis of these considerations, we assumed that pharmacological inhibition of the P2Y₁ receptor with the current agents may not efficiently and reliably inhibit thrombosis in vivo. In our effort to design anti-P2Y₁R agents, we hypothesized that an antibody targeting the ligand-binding site within the EL2 of the ADP/P2Y₁R (ie, amino acids T192-F215) can be used as a function-blocking reagent for platelet activation. This hypothesis is based on the notion that the EL2 domain of the P2Y₁ receptor plays an important role in ADP-induced platelet activation.

Figure 5. Occlusion and tail bleeding time measurements in second extracellular loop (EL2) of the P2Y₁ receptor (EL2Ab)-treated mice. Mice were injected with IgG (12.5 ng), EL2Ab (12.5 ng), or MRS2500 (2 mg/kg). One hour post dosing, FeCl₃-induced thrombosis (A) and tail bleeding time (B) measurements were conducted as described in the Materials and Methods in the online-only Data Supplement. Each point represents the occlusion time/bleeding time of a single animal.
Consequently, the aim of the present study was to characterize the antiplatelet and antithrombotic properties of this novel, custom-made antibody targeted against the EL2Ab. Our study should also provide insight into the pharmacological and potential therapeutic relevance of EL2Ab, and perhaps further validate the concept that targeting the P2Y₁R remains a relevant and viable alternative or complement to other antiplatelet strategies.

Our initial characterization revealed that EL2Ab has the capacity to recognize the denatured and native forms of the P2Y₁R protein, in a specific manner (eg, no band was detected in the P2Y₁R−/− platelets). So, we next sought to examine the antiplatelet activity of EL2Ab in both human and murine platelets/models. Our results indicated that EL2Ab dose dependently inhibited ADP/P2Y₁R-mediated platelet aggregation, in human (in vitro) and in mouse platelets (in vitro and ex vivo), and that this inhibition was found to be commensurate with its blockade of GPIIb-IIIa activation. These results also established EL2Ab as the first function-blocking antibody against ADP/P2Y₁R. Moreover, the dose–response profiles, coupled with the Western blot and flow cytometry analysis, indicate that there are no species (human versus mouse) differences in the EL2Ab affinity/potency for inhibiting aggregation induced by ADP, at least under in vitro conditions. This is not surprising given that the mouse and human P2Y₁Rs are identical in 20 of the 24 amino acids of EL2.

We also found that EL2Ab inhibited separate in vitro platelet functional responses, namely dense and α granule secretion, as well as phosphatidyserine exposure. Given that these responses are also mediated via the P2Y₁R, we assessed whether EL2Ab may cross react with its receptor protein. However, our data argue against this possibility as evidenced by the lack of ability of EL2Ab to interfere ADP-induced reduction in forskolin-mediated increases in cAMP, unlike the P2Y₁R antagonist AR-C 66096. To investigate the mechanism of action of EL2Ab further, radioligand displacement binding studies were performed using a selective P2Y₁R antagonist. Our data showed that EL2Ab displaced [³H]MRS2500 from its P2Y₁R, unlike its preabsorbed form. These data clearly demonstrate that EL2Ab exerts its inhibitory effects by directly binding to P2Y₁R ligand-binding site, thereby inhibiting ADP from binding to and activating the receptor protein.

Finally, having established the capacity of EL2Ab to inhibit platelet function (in vitro and ex vivo), we next determined whether these effects would uphold under in vivo experimental models (eg, antithrombotic activity). Indeed, our data in the murine model indicated that, similar to the experimental P2Y₁R antagonist MRS2500, EL2Ab was found to prolong the time for thrombus occlusion, supporting its use as anti-thrombotic agent. However, EL2Ab was also found to prolong the tail bleeding time, in mice, indicating that it also impairs hemostasis, much like MRS2500.

Collectively, these findings support the notion that antibody interaction with the EL2 region specifically blocks aggregation mediated through the ADP/P2Y₁R pathway and that the P2Y₁R remains a relevant clinical target, as an alternative or a complement to current therapeutically used antiplatelet strategies. Because an Ab-based antiplatelet biological has previously been approved by the Food And Drug Administration (ie, the GPIIb-IIIa antagonist abciximab/ReoPro), it is possible that EL2Ab would also have clinical applications, alone or in combination with other antithrombotic agents, especially given the limitations of the commonly used thromboembolic therapy (eg, resistance and severe bleeding associated with aspirin or Plavix). Finally, the identification of a functionally active P2Y₁R sequence, ie, EL2, should significantly aid molecular modeling study predictions for organic derivatives, which possess in vivo activity. This is an important consideration because in spite of the clear involvement of P2Y₁R signaling in occlusive vascular disease, P2Y₁R antagonists are still the only clinically effective drugs for the prevention of ADP-induced platelet activation.

Acknowledgments

We thank the members of the Khasawneh laboratory for critical reading of the article.

Sources of Funding

This work was supported by the National Heart, Lung, And Blood Institute of the National Institutes of Health under Award Number R15HL115567 (to F.T. Khasawneh). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosures

None.

References

ing, and function. These studies also constitute the first investigation of antibody-based inhibition of P2Y1 receptor function in platelets and highlight its EL2 domain as a potential target site for therapeutic interventions. Results obtained from these studies should provide knowledge and lay down the foundation for defining new and novel therapeutic targets or agents for the therapeutic management of multiple thrombosis-based disease states. Thus, EL2Ab could have widespread (experimental and therapeutic) applications, perhaps even as a complement or alternative in combination therapies.
Characterization of a Novel Function-Blocking Antibody Targeted Against the Platelet P2Y₁ Receptor


Arterioscler Thromb Vasc Biol. 2015;35:637-644; originally published online January 15, 2015; doi: 10.1161/ATVBAHA.114.304509

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/3/637

An erratum has been published regarding this article. Please see the attached page for:
/content/35/10/e50.full.pdf

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
In the article by Karim et al, which appeared in the March 2015 issue of the journal (Arterioscler Thromb Vasc Biol. 2015;35:637–644. DOI: 10.1161/ATVBAHA.114.304509), a correction was needed.

The affiliation was not correctly noted for Fatima Z. Alshbool. The correct affiliation is as follows: Department of Pharmacology, Loma Linda University, Loma Linda, CA.

The authors apologize for the error.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/35/3/637.
Materials and Methods

Human blood donation has been approved by the Institutional Review Board (IRB) at Western University of Health Sciences, Pomona, CA and all the donors were asked to sign a written consent, and a subjects’ bill of rights.

Reagents and Materials

ADP, fibrinogen, forskolin and RO20-1724 were obtained from Sigma Aldrich (St. Louis, MO), Thrombin, stir bars and other disposables were from Chrono-Log (Haverton, PA), and U46619 was obtained from Cayman Chemical (Ann Arbor, MI). The EL2Ab, which is an anti-peptide rabbit polyclonal antibody targeted to the second extracellular loop (i.e., T/Threo^{192–F}/Phen^{215}), was custom generated by EZ-Biolab (Carmel, IN). FITC-conjugated Annexin V, anti–P-selectin, and PAC-1 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). MRS2500 and AR-C 66096 were obtained from Tocris Bioscience (Ellisville, MO). The commercial P2Y_{1R} antibodies were from Santa Cruz (Santa Cruz, CA) and Alamone labs (Jerusalem, Israel) and used for western blot and flow cytometry, respectively. Note: the Santa Cruz antibody did not produce useful data in the flow cytometry studies due to high degree of “non-specificity”, which prompted us to investigate a commercial Ab produced by Alomone Labs.

Animals

C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME), whereas the P2Y_{1R}^{-/-} mice were a generous gift from Drs. Sean Ward and Kenton Sanders, University of Nevada. Mice were housed in groups of 1–4 at 24°C, under 12/12 light/dark cycles, with access to water and food ad libitum. All experiments involving animals were perform in compliance with the institutional guidelines, and were approved by the Western University of Health Sciences Institutional Animal Care and Use Committee.

Preparation of Platelets

Mouse blood was collected from a ventricle and the citrated (0.38%) blood was mixed with phosphate-buffered saline, pH 7.4, and was incubated with PGI2 (10 ng/mL; 5 minutes), followed by centrifugation at 237 × g for 10 minutes at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483 × g for 10 minutes at RT. The pellets were resuspended in HEPES/Tyrode buffer (HT; 20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM MgCl_{2}, 0.4 mM NaH_{2}PO_{4}, 12 mM NaHCO_{3}, 5 mM d-glucose) supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_{2}. Platelets were washed and resuspended in HT (pH 7.4) without EGTA, apyrase, or PGI_{2}. Platelets were counted with an automated hematology analyzer (Drew Scientific Dallas, TX) and adjusted to the indicated concentrations.

Washed human platelets were prepared as described in Karim et al. Briefly, Blood was drawn from healthy volunteers and blood was mixed with phosphate-buffered saline, pH 7.4, and was incubated with PGI_{2} (10 ng/mL; 5 minutes), followed by centrifugation at 237 × g for 10 minutes at room temperature (RT). PRP was isolated in the presence of apyrase (0.37 U/mL) and PGI_{2} (10 ng/mL) by centrifugation at 150 × g for 10 minutes at RT. PRP was centrifuged at 900 × g for 10 minutes and platelets were resuspended in HT containing 1 mM EGTA, apyrase, and PGI_{2}. Platelets were washed and resuspended in HT (pH 7.4) without EGTA, apyrase, or PGI_{2}.

In vitro Platelet Aggregation

PRP was incubated with the P2Y_{1R} antibody (EL2Ab) for 5 min before agonist stimulation (5 μM ADP, 1 μM U46619, 0.1 U/ml thrombin, or 5 μg/ml collagen). Platelet aggregation was measured by the turbidometric method using models 490 or 700 aggregometry systems (Chrono-Log Corporation, Haverton, PA). Each experiment was repeated at least 3 times, with blood collected.
from three different human donors, or from blood pooled from at least three separate groups of eight mice.

**Ex vivo Platelet Aggregation**
Mice were injected with normal rabbit IgG, or EL2Ab (12.5 ng) using the tail-vein (IV) route and blood was collected after one hour post injection. Platelets (with counts adjusted as described before) were stimulated with 5 µM ADP or 1 µM U46619, and aggregation was measured. Each experiment was repeated at least 3 times, with blood pooled from at least three separate groups of eight mice.

**[3H]MRS25000 displacement binding in intact platelets**
Resuspended platelets (1 x 10⁹ platelets/ml) were incubated with 1 nM [³H]MRS25000 at RT for 10 min, and then increasing concentrations of the displacing EL2Ab (1 ng-15 ng) were added for an additional 45 minutes. Next, the [³H]MRS25000 bound platelets were captured by running through 0.45 micron Millipore filters over a vacuum suction unit. The filters were then washed once and counted for radioactivity in a Beckman LS 6000 liquid scintillation counter. To calculate the non-specific binding, the same concentration of radio ligand was competed against 1000-fold excess of unlabeled MRS25000.

**EL2Ab characterization**

**Flow cytometry:** In order to characterize the ability of the EL2Ab to recognize the native form for the P2Y₁ receptor, flow cytometry studies were performed. These experiments were conducted as described before²,³,⁴. PRP from human or mouse origin was treated with 1 mM aspirin and 40 nM PGI₂, pH 6.5. The PRP was then spun down at 980 x g for 15 minutes to pellet the platelets. The supernatant (platelet free plasma) was discarded and the platelets were gently resuspended in buffer (138 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 5.5 mM glucose, 25 mM Tris-HCl and 40 nM PGI₂; pH 7.4) to a cell count of 1 x 10⁹ platelets/mL in the presence of 270 nM PGI₂. Fifty µl of cell suspension was placed in a flow cytometry tube and incubated with the EL2Ab (1:50 [v/v]), preabsorbed EL2Ab (1:50 [v/v]), normal rabbit IgG (control; 1:50 [v/v]), or an Ab against an extracellular domain of the P2Y₁ receptor (Alamone Labs; 1:50 [v/v]) as a positive control for 1 hour at room temperature. Next, 0.5 mL Phosphate-buffered Saline (PBS) was added and cells were washed by centrifugation for 5 minutes at 1200 rpm. The supernatant was aspirated, and this procedure was repeated at least twice. Fifty µl of the secondary FITC-conjugated goat anti-rabbit IgG was added (1:50 [v/v]) and incubated with the cells in darkness for 1 hour at room temperature. PBS (0.5 mL) containing 0.2% BSA was then added, and single-color analysis was performed on the samples using a Becton Dickinson Accuri™ C6 Flow Cytometer. A lower limit threshold was set for data acquisition, thereby eliminating background scatter. Analysis of flow profile was performed using the CFlow software.

**Western blot analysis:** Western blot was carried out as described in Karim et al.¹. Briefly, human or mouse platelets were lysed in 5x sample buffer and boiled at 95°C for 5 min. Finally protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P PVDF membranes (Bio-Rad, Hercules, CA). They were then probed with the primary antibody (EL2Ab) and visualized with horseradish peroxidase-labeled anti-rabbit IgG. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

**Platelet functional responses:**
Flow cytometric analysis was carried out as discussed in Lin et al.⁵. Briefly, human platelets (2 x 10⁸) were incubated in the presence or absence of P2Y₁ antibody (12.5 ng), for 5 minutes and
then stimulated with ADP (1 µM), U46619 (1 µM) for 3 minutes. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. Finally, platelets were incubated with FITC-conjugated Annexin V, anti-P-selectin, or PAC-1 antibodies at room temperature for 30 min in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/Tyrode buffer (pH 7.4). The samples were transferred to FACS-tubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFow Plus (BD Biosciences, Franklin Lakes, NJ).

**cAMP Measurements**

The cAMP assay was conducted as we described before6,7. Human PRP (500 µl) samples are collected and incubated with or without 12.5 ng of EL2Ab, or the P2Y₁₂ antagonist AR-C 66096 (1 µM) as a control. Next, platelets are treated with 0.5 µM forskolin before the addition of 5 µM ADP, and incubated at room temperature for 1 min. Next, the phosphodiesterase inhibitor RO20-1724 (100 µM) is added, and platelets spun down and pellet snap frozen in liquid nitrogen and stored at -70°C. Upon use, the pellet is resuspended in sodium acetate buffer (50 mM; pH 4.0), sonicated, boiled for 4 min, centrifuged, and the supernatant transferred to a new tube. The concentration of cAMP in the supernatant is measured according to a procedure described by Gilman6. Platelet samples with vehicle treatment are used to evaluate basal cAMP concentrations. The standard curve samples were prepared by adding known concentrations of cAMP to the supernatant from vehicle treated platelets.

**In vivo Thrombosis Model**

These studies were performed as described previously5. Briefly, mice 8–10 weeks old received IV injections of EL2Ab (12.5 ng), and were anesthetized with isoflurane. Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic micro-flowprobe (0.5 mm, Transonic Systems Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride (FeCl₃) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. Blood flow was continuously monitored for 45 min, or until blood flow reached stable occlusion (zero blood flow for 2 min). Data was recorded and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with FeCl₃). An occlusion time of 45 min was considered as the cut-off time for the purpose of statistical analysis.

**Tail Bleeding Time**

Mice were IV injected with EL2Ab (12.5 ng) or vehicle and the tail bleeding assay was performed after 1 hour. Hemostasis was examined using the tail transection technique5. Briefly, mice were anesthetized with isoflurane and place on a 37°C homeothermic blanket and their tails were transected 5 mm from the tip. The tail was placed in saline at 37°C and the time to blood flow cessation was measured. Clotting was not considered complete until bleeding had stopped for 1 minute. When required, measurements were terminated at 15 minutes.

**Statistical Analysis**

All experiments were performed at least three times. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SEM. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using t-test, and similar results were obtained. Significance was accepted at P<0.05 (two-tailed P value), unless stated otherwise.
Supplemental References:


