Role of P2Y receptor subtypes in platelet-derived microparticle generation

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1. ABSTRACT

Microparticles are shed from the platelet membrane upon platelet activation by strong agonists, and they aid in clot formation. As the P2Y1 and the P2Y12 receptors differentially contribute to different platelet functions, we studied the relative contribution of the P2Y1 and P2Y12 receptors to microparticle formation from platelets. The P2Y12 receptor antagonist AR-C 69931MX, but not the P2Y1 receptor antagonist MRS2179, caused a significant decrease in the number microparticles formed by convulxin and thrombin. In addition, there was no significant decrease in microparticle formation in P2Y1 knockout mouse blood when compared to the wild type mice. These results illustrate that the P2Y12 receptor contributes to microparticle formation from activated platelets by a strong agonist, without any significant involvement of the P2Y1 receptor. We also conclude that there is no correlation in the number of microparticles circulating in vivo between the P2Y1 receptor null mice and the wild type mice under unstimulated conditions. Finally, we conclude that the increased bleeding time in the P2Y1 null mice is due to overall platelet dysfunction and not due to the decrease of circulating microparticles.

2. INTRODUCTION

Injury to a blood vessel results in the exposure of subendothelial collagen and the initiation of the coagulation cascade leading to the production of thrombin and fibrin. Thrombin-mediated platelet activation occurs through protease activated receptors (PAR) 1 and 4. Both receptors initiate intracellular signaling through the activation of G proteins Gq and G12/13. Collagen, the other major physiological agonists, activates platelets through the tyrosine kinase receptor, glycoprotein VI. Activation by thrombin and collagen cause platelets to shed components of their plasma membranes into the blood vessel, these fragments are known as microparticles (MP). These microparticles contain cytoplasmic elements and are rich in negatively-charged phospholipids such as phosphatidylserine, which can serve as a procoagulant substrate for tenase and prothrombinase complexes.

Microparticles are approximately 0.1 micrometers in diameter and have been implicated in being involved in inflammation, coagulation and vascular function (1-3). These small fragments are generated under conditions of high shear stress and platelet activation (4, 5).
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Microparticles are known as bioactive lipids formed by the breakdown of the membrane skeleton and surrounded by a phospholipid bilayer expressing the same adhesive molecules and proteins found on platelets (5-8). They contain negatively charged phospholipids such as phosphatidylycerine (PS) that facilitate the binding of activated coagulation factors (prothrombinase and tenase) to the membrane (9-11). Adhesion molecules such as GPIb and alphaIIb-beta3, which binds vWF and fibrinogen respectively, are present on the bioactive lipids as well (12). It is believed that tissue factor is not present on platelet- derived microparticles, however microparticle composition is still not well defined.

The P2Y receptors play an important role in hemostasis. Secreted ADP from dense granules has show to potentiate granule release, alphaIIbbeta3 activation and thrombin generation (13-15). It has previously been reported that the generation of platelet-derived microparticles are decreased in the presence of the P2Y12 receptor antagonist AR-C69931MX upon Gq and GPVI stimulation (16). It has also been shown that P2Y12 null mice have a bleeding deficiency (17). Therefore we investigated whether the P2Y1 null mice bleeding deficiencies can be correlated with microparticle generation. We studied the affect of antagonizing the P2 receptors on MP generation in human platelets and in P2Y1 receptor knockout mouse platelets.

Our results indicate that only the P2Y12 receptor may be involved in the formation of microparticles without substantial role for the P2Y1 receptor. We found no correlation between circulating microparticle number and the P2Y1 deficiency, and we conclude that the increased bleeding time is due to overall platelet dysfunction and not due to lack of microparticles.

3. MATERIALS AND METHODS

3.1. Materials

Apyrase (Type VII), bovine serum albumin (fraction V), thrombin, and acetylsalicylic acid were obtained from Sigma (St.Louis, MO). Convulxin was purchased from CenterChem Inc. (Norwalk, CT). CD41, CD42b, CD41b antibodies. P2Y1 deficient mice were generated in our laboratory and were described in a previous publication (13).

3.2. Isolation of human platelets

Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230 X g for 10 minutes. The platelet pellet was then centrifuged for 10 minutes at 37°C. The PRP was then centrifuged for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). PRP was incubated with 1 mM acetylsalicylic acid for 30 min at 37°C. The PRP was then centrifuged for 10 minutes at 980 X g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 3 mM NaH2PO4, 5 mM glucose, 10 mM Hepes, pH 7.4, 0.2% bovine serum albumin) containing 0.01U/ml apyrase. Cells were counted using the Coulter Z1 Particle Counter and concentration of cells was adjusted to 4X10^8 platelets/ml. All experiments using washed platelets were performed in the absence of extra cellular calcium unless otherwise mentioned.

3.2. Isolation of mouse platelets

Blood was collected from the vena cava of anesthetized mice into syringes containing 1/10th blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100 X g for 10 minutes. Platelet-rich plasma was recovered, and platelets were pelleted at 400 X g for 10 minutes. The platelet pellet was resuspended in Tyrode's buffer (pH 7.4) containing 0.01U/ml apyrase. The washed platelets were subsequently used for experiments.

3.3. Measurement of mouse bleeding times

Wild type, heterozygous and knockout mice were bled at 6-8 weeks old by cutting 0.3 cm from the end of the tail and then submerging it in 0.9% saline. Once the tail was cut a timer was started and continued to run until stoppage of bleeding. When no blood was seen dripping from the end of the tail the bleeding was deemed stopped and the time was recorded. Bleeding was automatically stopped once the time reached 30 minutes.

3.4. Aggregometry

Following the platelet preparations as described above, aggregation of 500 microliters washed platelets was analyzed using a P.I.C.A lumiaggregometer (Chrono-log Corp., Havertown, PA) measuring light transmission under stirring conditions (900rpm) at 37°C. Agonists were added for platelet stimulation, and on certain occasions the platelets were preincubated with isolated microparticles for 1 minute at 37°C. Each sample was allowed to aggregate for at least 3 minutes. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

3.5. Flow cytometry

All determinations were performed with an FASCA flow cytometer. Whole blood and washed platelets were analyzed prior to and following activation with thrombin (1U/ml) and convulin (100 ng/ml) with and without the presence of MRS2179 (100 micromolar) and/or AR-C69931MX (100 nM). Surface expression of CD41 and CD42 were examined.

Washed platelets were prepared as described above whereas whole blood samples were diluted 1:10 in Tyrode’s buffer. Both sets of platelets were separated into 50 μl aliquots. Following addition of inhibitors and stimulation with thrombin and convulxin, the platelets were incubated for 20 minutes in the dark at room temperature with 5 microliters of the platelet specific GPIb antibody CD41-FITC or the GPIb andtibody CD42-PE. In whole blood the synthetic tetrapeptide GPRP (2.5 mmol/l) was added to prevent fibrin polymerization. Samples were analyzed within 1 hour by flow cytometry and platelets and microparticles were identified based on particle size (forward scatter) and complexity (side scatter). Light
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Figure 1. Flow cytometry of washed platelets and microparticles. Washed human platelets were labeled with the GPIIb antibody and measured by FSCSA analysis under unstimulated (A) or stimulated (B) conditions. Gate “R1” is represents the location of the platelets, and gate “R2” is representative of the location of microparticles. Generation of microparticles was obtained by stimulating platelets with thrombin (1 U/ml) and convulxin (100 ng/ml). The data are representative of at least three independent experiments with platelets from at least three donors.

3.6. Preparation of microparticles
Washed platelets were prepared as previously described. Platelets were resuspended at $10^8$ concentration. They were then allowed to aggregate with thrombin (1 U/ml) and convulxin (100 ng/ml) for 10 minutes. Following stimulation the platelets were spun down at 1500 g for 15 min. The supernatant containing the microparticles was removed and spun at 13,000 g for 30 min at 4°C. The pellet containing the microparticles was then resuspended with Tyrode’s buffer. The wash was repeated two more times and the microparticles were resuspended and used in aggregation.

4. RESULTS
4.1. Effect of P2Y receptor antagonists on platelet microparticle formation
Platelet microparticles are procoagulant bodies generated from platelets upon stimulation by strong agonists such as thrombin or collagen, while weaker agonists such as ADP do not generate microparticles when presented as a single stimulus. However, the relative contribution of the platelet P2Y receptors to the microparticle formation by strong agonists has not been thoroughly studied. We evaluated the relative contribution of the P2Y1 and the P2Y12 receptors using the selective P2Y1 receptor antagonist, MRS2179, and the P2Y12 receptor antagonist AR-C69931MX. Washed platelets were labeled with the platelet specific GPIIb antibody, stimulated with thrombin and convulxin and analyzed by flow cytometry. Figure 1 depicts a sample tracing of the flow cytometry diagram under unstimulated and stimulated conditions. Gate “R1” represents the area of platelets and gate “R2” represents the microparticles. The forward and side scattering designations are consistent with previous studies (5, 16, 18). Unstimulated platelets exhibit a low response in gate “R2” (Figure 1A), whereas platelets stimulated with thrombin (1U/ml) and convulxin (100 ng/ml) display a large increase “R2” observed by flow cytometry (Figure 1B). The increase in components in gate “R2” upon agonist stimulation is consistent with previous studies on platelet-derived microparticles (5, 16, 18). The microparticles were counted as a percent compared to that under maximal stimulation and plotted accordingly.

Washed human platelets were treated with thrombin and convulxin in the presence and absence of the P2Y receptor antagonists. Thrombin and convulxin–mediated microparticle generation from washed platelets was significantly inhibited when treated with the P2Y12 receptor antagonist, AR-C69931MX compared to the control (p<0.05). However, there was no significant inhibition of microparticle generation in the presence of the P2Y1 receptor antagonist, MRS2179 (p>0.05) (Figure 2).

4.2. Effect of microparticles on agonist-induced platelet aggregation
Microparticles were generated from human washed platelets by thrombin and collagen activation as described in earlier publications (19, 20), and added to 500 microliters of washed platelets. The addition of microparticles to the washed platelet suspension did not have any effect on the platelets upon addition for up to 1 minute prior to stimulation with an agonist. Subsequently, platelets were stimulated with the thromboxane analog, U46619, at maximal and submaximal conditions in the presence and absence of microparticles. Our results indicate that the presence of microparticles at submaximal concentrations of U46619 (300 nM) potentiates the aggregatory response (Figure 3). However, platelet aggregation did not differ in platelet suspensions containing microparticles when stimulated with the maximal concentration of U46619 (1micromolar). This indicates that at lower concentration of an agonist, such as U46619, microparticles may potentiate the platelet response, however there is no significant effect of microparticles on the maximal concentration of U46619-induced platelet aggregation.

4.3. Effect of ADP receptor antagonists on microparticle generation from mouse platelets
Mice deficient in either the P2Y1 or the P2Y12 receptors have increased bleeding times (>30 min) (17, 21,
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Figure 2. ADP receptor antagonists inhibit the generation of platelet-derived microparticles. Washed human platelets were stimulated with 1 U/ml Thrombin and 100 ng/ml convulxin in order to generate microparticles. Samples were stimulated in the absence or presence of the P2Y1 receptor antagonist MRS2179 (100 micromolar) or the P2Y12 receptor antagonist AR-C69931MX (100 nM). Microparticle generation was collected by FACSA and calculated as a percent of maximal stimulation. The data are representative of at least three independent experiments with platelets from at least three donors.

Figure 3. Platelet aggregation is potentiated in the presence of platelet-derived microparticles at submaximal concentrations of U46619. Washed human platelets were stimulated at maximal and submaximal concentrations of the TP receptor agonist U46619 and incubated in the presence or absence of platelet-derived microparticles. The data are representative of three independent experiments with platelets from three different donors.

We have generated the P2Y1 deficient mice in our laboratory (23) and confirmed that they have a bleeding deficiency (data not shown) consistent with previous reports (21, 22). Microparticles have been implicated in having an important role in hemostasis. The hypothesis in question is whether the bleeding deficiency observed with the absence of the P2Y receptor is due to a decrease in circulating microparticles. Our studies have shown that addition of microparticles can increase platelet aggregation by submaximal stimulation with agonists. We thus investigated whether the increase in bleeding time was due to an overall decrease in microparticle generation. Washed platelets from C57BL6 mice were stimulated with thrombin and convulxin in order to generate platelet-derived microparticles. P2Y receptor antagonists, MRS2179 and AR-C69931MX, inhibited microparticle generation compared to that of platelets treated with only the agonists (Figure 4). Consistent with the abovementioned human data, P2Y12 receptor antagonist AR-C69931MX significantly inhibited microparticle generation (p<0.05) whereas the P2Y1 receptor antagonist did not (p>0.05).

Finally, P2Y1 knockout mice were used to investigate whether there is any difference in the amount of circulating platelet-derived microparticles between the wild type mice and the P2Y1 receptor null mice. Microparticles were counted in whole blood under unstimulated conditions. Therefore these results relate to circulating microparticles in vivo under physiologic conditions. The microparticles present in unstimulated whole blood was compared and normalized to generated microparticles in whole blood under stimulated conditions. Our findings indicate that there is no statistically significant difference in microparticle number between wild type and P2Y1 null mice in whole blood (Figure 5) indicating the P2Y1 receptor deficiency does not lead to a decrease in circulating platelet-derived microparticles.

5. DISCUSSION

Microparticles are bioactive lipids that may play a role in platelet activation. Using knockout mice and P2Y receptor antagonists we studied whether microparticles had any potentiating affect on aggregation. Finally, we investigated whether a correlation exists between the bleeding times and the number of in vivo-circulating platelet-derived microparticles in the P2Y1 receptor null mice.

The results indicate that P2Y12 receptor antagonists, but not the P2Y1 receptor antagonists, inhibit microparticle generation upon stimulation of platelets downstream of PARs and GPVI. Microparticle generation was inhibited in both human and mouse washed platelets upon activation in the presence of these antagonists. Interestingly, unstimulated mouse platelets yielded a high percentage of microparticles possibly due to the harsh conditions the platelets are exposed to during their isolation. The blood is removed from the mice by the heart puncture technique and repeatedly centrifuged. The removal of the blood from the heart along with repeated centrifugations may cause an increase in platelet-derived microparticles in response to stress on the platelets.

Our studies are in agreement with the report by Takano et al, who have shown that microparticle generation
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**Figure 4.** ADP receptor antagonists inhibit the generation of platelet-derived microparticles in mouse platelets. Washed mice platelets were stimulated with 1 U/ml thrombin and 100 ng/ml convulxin in order to generate microparticles. Samples were stimulated in the absence or presence of the P2Y1 receptor antagonist MRS2179 (100 micromolar) or the P2Y12 receptor antagonist AR-C69931MX (100 nM). Microparticle generation was collected by FACS and calculated as a percent of maximal stimulation. The data are representative of at least three independent experiments with platelets from at least three donors.

**Figure 5.** There is no statistically significant difference in microparticle formation between wild type and P2Y1 null mice platelets. Whole blood from wild type and knockout mice was analyzed under unstimulated conditions and the presence of microparticles was compared to microparticles generated under stimulated conditions (1 U/ml thrombin and 100 ng/ml convulxin). The data are representative of at least three independent experiments with platelets from at least three donors.

with ADP and collagen was significantly decreased in the presence of P2Y12 antagonists (16). This study goes beyond the conclusions of Takano et al and demonstrates that secreted ADP upon thrombin plus collagen stimulation significantly contributes to the microparticle generation from platelets.

Secondly, we investigated whether microparticles enhanced platelet aggregation. Microparticles were generated by the addition of thrombin to the washed platelet suspension and subsequently collected separate from the platelets. Washed platelets were stimulated with the thromboxane analog U46619 in the presence or absence of the microparticle suspension. Following the addition of the microparticle suspension, platelets were allowed to sit for at least one minute prior to stimulation to avoid the possibility of activation by residual thrombin or secreted ADP present in the microparticle suspension. At submaximal concentrations of U46619, there was a potentiation of aggregation by exogenous microparticles. These results indicate that platelet-derived microparticles may have some role in the activation of platelets in vivo.

The P2Y1 receptor null mice developed in our lab exhibit a bleeding phenotype as reported by others (17, 21, 22). As described above, we have shown that platelet-derived microparticles can potentiate platelet aggregation, therefore we investigated whether the bleeding phenotype would correlate with a decreased number of microparticles released from platelets. We found no statistically significant difference in the number of circulating platelet-derived microparticles between the wild type and knockout mice indicating that the bleeding phenotype is due to an overall platelet deficiency and a critical role of the P2Y1 receptor in thrombus growth and stability.

In conclusion, our study demonstrates that the P2Y12 receptor, but not the P2Y1 receptor, contributes to microparticle formation from the activation of platelets by strong agonists. Interestingly, microparticles potentiate low concentration agonist-induced platelet aggregation indicating a possible role in activating platelets during thrombosis and hemostasis. We further conclude that there is no correlation between the number of circulating platelet-derived microparticles in vivo and the bleeding diathesis in the P2Y1-deficient mice. We propose that the P2Y12 receptor blocking drugs will have enhanced potency to reduce thrombosis by reducing the formation of platelet-derived microparticles in vivo, which we have shown to potentiate platelet activation.

6. REFERENCES


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**Abbreviations:** PAR: protease activated receptors; MP: microparticles; PS: phosphatidylserine; GPIb: glycoprotein Ib; vWF: von Willebrand factor; Thr: thrombin; Cvx: convulxin; GPVI: glycoprotein VI; ADP: adenosine diphosphate

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