Vessel wall, not platelet, P2Y$_{12}$ potentiates early atherogenesis

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Aims

Platelets have a fundamental role in atherothrombosis, but their role in early atherogenesis is unclear. The P2Y$_{12}$ receptor is responsible for amplifying and sustaining platelet activation and P2Y$_{12}$ inhibition is crucial in modulating the vessel wall response to injury. We therefore examined the role of platelet vs. vessel wall P2Y$_{12}$ in early atherogenesis and considered the use of P2Y$_{12}$ antagonists ticagrelor and clopidogrel in modulating this process.

Methods and results

ApoE$^{-/-}$ and ApoE$^{-/-}$/P2Y$_{12}^{-/-}$ male mice underwent bone marrow transplantation and were fed a western diet for 4 weeks before assessing atherosclerotic burden. Compared with ApoE$^{-/-}$ controls, platelet P2Y$_{12}$ deficiency profoundly reduced platelet reactivity but had no effect on atheroma formation, whereas vessel wall P2Y$_{12}$ deficiency significantly attenuated atheroma in the aortic sinus and brachiocephalic artery (both $P < 0.001$). ApoE$^{-/-}$ and ApoE$^{-/-}$/P2Y$_{12}^{-/-}$ male mice fed western diet plus either twice-daily doses of ticagrelor (100 mg/kg) or daily clopidogrel (20 mg/kg) for 4 weeks exhibited no significant reduction in atheroma compared with control mice fed mannitol. Attenuated P-selectin expression confirmed platelet P2Y$_{12}$ inhibition in drug-treated mice.

Conclusions

Despite its major contribution to platelet reactivity, platelet P2Y$_{12}$ has no effect on early atheroma formation, whereas vessel wall P2Y$_{12}$ is important in this process. Ticagrelor and clopidogrel effectively reduced platelet reactivity but were unable to inhibit early atherogenesis, demonstrating that these P2Y$_{12}$ inhibitors may not be effective in preventing early disease.

Keywords

Platelet  •  P2Y12  •  Atherogenesis  •  Ticagrelor  •  Clopidogrel

1. Introduction

Atherosclerosis is a chronic inflammatory disease, involving a complex network of cellular processes, and disease progression is well defined,$^{1–4}$ yet novel cell roles continue to emerge. In addition to their fundamental role in atherothrombosis, platelets are known to interact with the endothelium and leucocytes and, upon activation, can elicit an inflammatory response in these cells via both direct adhesion and the release of pro-inflammatory cytokines from platelet α-granules.$^{5–7}$ Furthermore, activated platelets can induce mitogenic effects on the vessel wall through secretion of growth factors.$^{8,9}$ With their pro-atherogenic arsenal, platelets are increasingly regarded as discrete immune cells, yet the degree to which they contribute directly to plaque development in the early stages of disease remains to be fully established. Several studies have investigated various aspects of platelet involvement, including adhesion receptors and proteins,$^{10–13}$ alluding to the notion that platelet activation potentiates lesion development.

Platelets possess receptors for many agonists, including thrombin, that initiate a platelet activation response and this is then amplified and sustained by the G protein-coupled receptor P2Y$_{12}$. Consequently, inhibition of P2Y$_{12}$ leads to a significant attenuation of both platelet aggregation and granule secretions.$^{14}$ As a result, P2Y$_{12}$ inhibitors, such as ticagrelor and clopidogrel, are essential in the treatment and prevention of thrombotic events in acute coronary syndromes.$^{15–18}$ In addition, platelet P2Y$_{12}$ inhibition has been shown to be crucial in modulating the vessel wall response to injury.$^{19,20}$ Although predominantly expressed on platelets, P2Y$_{12}$ is also expressed on vascular smooth muscle cells (VSMCs), where it mediates cell contraction$^{21}$ and promotes pro-inflammatory and mitogenic responses via a thrombin-induced pathway.$^{22}$ Recent work has suggested that platelet P2Y$_{12}$ and possibly vessel wall P2Y$_{12}$ play a role in the development of mature atherosclerotic plaques in a mouse atherosclerosis model.$^{23}$ Given this evidence, we hypothesized that P2Y$_{12}$ activation may be important in promoting early atherogenesis, in contrast to later stages of disease.
when endothelial disruption may promote the role of platelets. In this study, we investigated the role of P2Y12, using a mouse model of atherogenesis and employed bone marrow transplantations to examine the contributions of platelet vs. vessel wall P2Y12 in early lesion formation.

In addition, we considered the application of ticagrelor and clopidogrel treatment in the modulation of atherogenesis. Recent data show that ticagrelor also inhibits adenosine re-uptake by erythrocytes and blocks ADP-induced vasoconstriction in isolated arteries. Furthermore, ticagrelor offers a reduced mortality risk, compared with clopidogrel, in patients with acute coronary syndromes but the mechanisms for this mortality reduction remain to be established. We therefore also aimed to determine any non-P2Y12-mediated effects of ticagrelor or clopidogrel on atherogenesis.

2. Methods

Expanded methods including genotyping, bone marrow cell preparation, and flow cytometry can be found in Supplementary material online.

2.1 Animals

Transgenic mice were obtained from in-house colonies derived from breeding pairs sourced externally. ApoE−/− mice (JAX 2052) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and P2Y12−/− mice were derived from breeding pairs provided by Schering Plough Research Institute, NJ (courtesy of Dr M. Chintala). Both strains were developed on a C57BL/6 background and backcrossed 10 generations. ApoE−/− P2Y12−/− (DK) mice were generated from a cross of each parent colony and offspring were genotyped by polymerase chain reaction (PCR).

Male mice were used for all experiments to avoid sex-related differences on atherogenesis. These were fed a standard chow diet (Harlan Laboratories, Indianapolis, IN, USA) until the commencement of each procedure aged 8–10 weeks, weighing ≏25 g. At the end of each procedure, mice were euthanized by an overdose of sodium pentobarbital via intraperitoneal injection. Experiments were performed in accordance with UK and European legislation under the Animals (Scientific Procedures) Act 1986 and European Directive 2010/63/EU.

2.2 A murine model of early atherogenesis

Atherosclerosis was augmented by feeding male mice a high-fat (21%) western diet (829100; Special Diet Services, Braintree, UK) for 4 weeks, after which tissues were assessed for atherosclerotic burden. Bone marrow transplanted mice were allowed to recover for 5 weeks on a chow diet prior to switching to western diet. Animals were perfusion-fixed and embedded into paraffin. The brachiocephalic artery and aortic sinus were fixed for histological examination and employed bone marrow transplantations to examine the contributions of platelet vs. vessel wall P2Y12 in early lesion formation.

Bone marrow transplantations involved bone marrow P2Y12 phenotype was confirmed by mannitol. All mice were observed daily to monitor jelly consumption and efficacy of drug delivery was determined by P-selectin expression in response to PAR-4 TRAP.

2.8 Platelet P-selectin and activated GPIIb/IIIa receptor expression in diluted whole blood

Blood was taken by cardiac puncture into hirudin (50 µL/mL, final concentration) before diluting in HEPES-Tyrode's (HT) buffer with calcium chloride.
Vessel wall P2Y12 potentiates early atherogenesis

Aliquots of dilute blood were incubated with FITC anti-CD62p (BD Biosciences, Oxford, UK), PE anti-CD41/61 JON/A (Em fret Analytics, Eibelerstadt, Germany), and increasing concentrations of PAR-4 TRAP (1–3 mmol/L; AYPGKF; Almac Sciences, East Lothian, UK) for 20 min at room temperature and analysed by flow cytometry. Expression was denoted as increase in median fluorescence of the entire platelet population over baseline values.

2.9 Coagulation tests

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured to assess the intrinsic (aPTT) and extrinsic (PT) coagulation pathways. Fibrinogen levels were a measure of the final common coagulation pathway. Blood was taken by cardiac puncture into 3.8% (final w/v) sodium citrate at a ratio of one part anticoagulant to nine parts blood, as used in previous murine studies.30 Samples were analysed by the Coagulation Department at the Royal Hallamshire Hospital (Sheffield Teaching Hospitals NHS Foundation Trust, UK).

2.10 Statistical analysis

Data are expressed as mean or individual values and presented with SEM. Independent samples’ t-test and one- or two-way ANOVA were performed with Tukey’s multiple comparison test using Prism (Version 6; GraphPad, San Diego, CA, USA) or SPSS software (Version 19; IBM, New York, NY, USA).

3. Results

3.1 Generation of an apoE−/− P2Y12−/− colony

To investigate the role of P2Y12 in early atherogenesis, a double-knockout ApoE−/− P2Y12−/− (DK) mouse model was created from crossing in-house ApoE−/− and P2Y12−/− parent colonies. P2Y12 genotype was determined by PCR and further confirmed by a functional assay measuring platelet P-selectin and activated GPIIbIIa receptor expression in response to PAR-4 TRAP. Platelets from DK mice demonstrated significantly attenuated P-selectin expression (P < 0.05 vs. ApoE; Figure 1A) and a reduction in the activated form of the GPIIbIIa receptor (P < 0.001 vs. ApoE; Figure 1B) compared with ApoE−/− mice in response to 1 mmol/L PAR-4 TRAP. A similar response was also seen at the higher concentration of TRAP. These results were consistent with our previous findings on P2Y12−/− mice.19

3.2 Vessel wall, not platelet, P2Y12 plays a role in early atherogenesis

In order to assess the contribution of platelet and vessel wall P2Y12 in early atherogenesis, bone marrow transplantations were performed. In addition to ApoE−/− and DK control groups, mice deficient in either platelet or vessel wall P2Y12 were generated. Thoracic aortae stained with Oil Red O demonstrated a non-significant trend towards reduced % atheroma in vessel wall P2Y12−/− deficient mice (Figure 2A and C). However, when assessing the aortic arch alone, atheroma was significantly attenuated in these mice (Figure 2B): aortic arch lesion area was 6.7 ± 0.83% in control mice, 4.33 ± 0.4% in mice with deficient vessel wall and platelet P2Y12 (P < 0.05 vs. control), and 4.4 ± 0.54% in mice with only deficient vessel wall P2Y12 (P < 0.05 vs. control). There was no significant effect of vessel wall P2Y12 in the descending aortae (Figure 2B). Mice with absence of only platelet P2Y12 exhibited no differences in lesion area compared with controls.

A similar pattern was repeated in other areas of the arterial tree in mice whose vessels lacked P2Y12, an effect which was irrespective of platelet P2Y12 expression. In the brachiocephalic artery, lesion area, represented as a ratio of the CSA, was 0.027 ± 0.002 in control mice, 0.004 ± 0.003 in mice with deficient vessel wall and platelet P2Y12 (P < 0.001 vs. control), and 0.006 ± 0.003 in mice lacking only vessel wall P2Y12 (P < 0.001 vs. control) (Figure 2D). Absence of platelet P2Y12 alone appeared to have no effect on lesion size (0.021 ± 0.004; P = 0.56 vs. control). Analogous results were observed in the aortic sinus with notably less atheroma observed in mice deficient in vessel wall P2Y12 (Figure 2E and F). Aortic sinus lesion area was 0.071 ± 0.008 in control mice, 0.024 ± 0.003 in mice with deficient vessel wall and platelet P2Y12 (P < 0.001 vs. control), and 0.03 ± 0.006 in mice lacking only vessel wall P2Y12 (P < 0.001 vs. control). MSB and α-smooth muscle actin staining for collagen and smooth muscle cells, respectively, within these aortic sinus lesions demonstrated little positive staining in those mice deficient in vessel wall P2Y12. In contrast, mice which did express vessel wall P2Y12 demonstrated much greater staining for both collagen and α-smooth muscle actin regardless of platelet P2Y12 expression (see Supplementary material online, Figures S1 and S2).

The method employed in this study for bone marrow transplantation has successfully been used previously by our group.19,26 We further confirmed the bone marrow P2Y12 phenotype and therefore assessed the efficacy of transplantation by measuring P-selectin expression and the presence of activated GPIIbIIa in response to PAR-4 TRAP in platelets from transplanted ApoE−/− mice. Control mice transplanted with ApoE−/− bone marrow expressed high levels of P-selectin and activated GPIIbIIa over baseline values in response to PAR-4 TRAP (see Supplementary material online, Figure S3). This response, however, was considerably attenuated in ApoE−/− mice transplanted with DK bone marrow.
and thus confirmed the successful reconstitution of P2Y12-deficient donor bone marrow into recipient mice. In addition, we measured PT, aPTT, and fibrinogen levels to determine any variation in the potential for thrombin and fibrin generation between bone marrow transplanted mice and found no differences in these coagulation parameters (see Supplementary material online, Figure S4).

### 3.3 Ticagrelor and clopidogrel have no effect on early atherogenesis

ApoE−/− and DK male mice were fed either ticagrelor, clopidogrel, or mannitol (control) for 4 weeks, in addition to a western diet, to investigate the effects of pharmacological inhibition of P2Y12 on early atherogenesis.

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**Figure 2** ApoE−/− and DK male mice underwent bone marrow transplantation to generate chimeric groups: (1) ApoE into ApoE, (2) DK into ApoE, (3) DK into DK, and (4) ApoE into DK. Following 4 weeks on a western diet, aortae (n = 12, 15, 12, and 12, respectively) were stained with Oil Red O (C) and lesion area was calculated as a percentage of surface area for the whole aorta (A) and the aortic arch and descending aorta (B). The brachiocephalic artery (D) (n = 10, 13, 8, and 9) and aortic sinus (E) (n = 13, 14, 9, and 10) were sectioned and stained with Alcian Blue/Elastic Van Gieson (F). Lesion area was quantified from at least five sections per mouse. Data are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 (A, D and E: one-way ANOVA with Tukey’s multiple comparison; B: two-way ANOVA with Tukey’s multiple comparison).
Vessel wall P2Y<sub>12</sub> potentiates early atherogenesis

ApoE delivery, we measured P-selectin expression in response to 1 mmol/L PAR-4 TRAP. In this study, we identify a previously unknown role of vessel wall P2Y<sub>12</sub> in modulating early atherogenesis and therefore propose that it is vessel wall P2Y<sub>12</sub>, plays a profound role in the vessel wall response to injury.3 This is also true of the study by Li et al. which assessed atheroma in ApoE<sup>−/−</sup>P2Y<sub>12</sub><sup>−/−</sup> (DK) mice after 20 weeks on a high-fat diet, similar in composition to our own western diet.23 We chose to study a much earlier stage of atherogenesis, after only 4 weeks of feeding, where we could both accurately measure atheroma and also be confident of little risk of endothelial erosion. From our results, we see that it is vessel wall, rather than platelet, P2Y<sub>12</sub> which plays a significant role in the early stages of disease by promoting lesion development. Furthermore, we found that P2Y<sub>12</sub> antagonists, ticagrelor and clopidogrel, were unable to inhibit these effects and so therefore may not offer a therapeutic strategy for the prevention of atherosclerosis in people without evidence of atherosclerotic disease.

The pro-inflammatory capacity of platelets is evident and many studies have been conducted to examine their potential role in propagating lesion development, implicating roles for platelet adhesion proteins and receptors including P-selectin, GPIb, and VI.10 In addition, the deposition of cytokines, such as RANTES (CCL5) and platelet factor 4 (CXCL4), by activated platelets onto the endothelium has been attributed to increased leucocyte recruitment to atherosclerotic and injured vessels.5,13,31 With its central role in platelet reactivity, the P2Y<sub>12</sub> receptor is an attractive candidate for investigation. In a recent study, Li et al. present evidence to suggest platelets promote lesion development via P2Y<sub>12</sub>-mediated granule release of P-selectin and platelet factor 4, thus amplifying leucocyte recruitment.23 Yet, despite this apparent role, we observed no such effect of platelet P2Y<sub>12</sub> in our own model of early atherogenesis.

To our knowledge, all platelet atherogenesis studies to date model established disease, choosing end points from 7 to more than 30 weeks on a chow or high-fat diet.10–12,32 Cholesterol clefts and fibrous caps are common features in these lesions and are typically associated with advanced plaques, including those which are susceptible to endothelial erosion and rupture.3 This is also true of the study by Li et al. which assessed atheroma in ApoE<sup>−/−</sup>P2Y<sub>12</sub><sup>−/−</sup> (DK) mice after 20 weeks on a high-fat diet, similar in composition to our own western diet.23 We chose to study a much earlier stage of atherogenesis, after only 4 weeks of feeding, where we could both accurately measure atheroma and also be confident of little risk of endothelial erosion. From our results, we see that it is vessel wall, rather than platelet, P2Y<sub>12</sub> which is important in the development of early lesions, but perhaps this balance shifts as disease progresses.

Figure 3 ApoE<sup>−/−</sup> and DK male mice were fed western diet for 4 weeks in addition to either twice daily doses of ticagrelor (100 mg/kg; n = 10 and 9, ApoE and DK, respectively), daily clopidogrel (20 mg/kg; n = 9 and 8) or mannitol (control; n = 9 in both groups). Whole aortae (A) and aortic sinus (B) lesion area were quantified. Data are mean ± SEM; ***p < 0.001 (Independent samples t-test); P = NS (two-way ANOVA with Tukey’s multiple comparison).

Figure 4 P-selectin expression (median fluorescence) in response to 1 mmol/L PAR-4 TRAP in platelets from ApoE<sup>−/−</sup> and DK male mice treated with either ticagrelor (n = 7 and 6, ApoE, and fD, respectively), clopidogrel (n = 5 and 6) or mannitol (control; n = 7 and 6). Data are mean ± SEM; ***p < 0.001 (two-way ANOVA with Tukey’s multiple comparison).
of vessel wall P2Y$_{12}$ on the results demonstrated by Li et al. Only DK mice were chosen as recipients for their bone marrow transplantations and so further investigation looking at the role of vessel wall P2Y$_{12}$ in later atherogenesis is warranted. Research into the role of the related platelet purine receptor P2Y$_1$ in atherogenesis, which is more widely expressed than P2Y$_{12}$, demonstrated a similar vascular wall effect on atherogenesis and so also supports the need for further exploration in this area. 

Although platelets are deemed to be the major source of P2Y$_{12}$ in the body, the receptor has also been identified in VSMCs. In order to delineate the separate roles of platelet and vessel wall P2Y$_{12}$ in early atherogenesis, we performed bone marrow transplantations, enabling the creation of chimeric mice expressing either vessel wall or platelet P2Y$_{12}$. We found that, in the absence of vessel wall P2Y$_{12}$, atheroma is attenuated throughout the arterial tree and appears to result in an earlier lesion phenotype with less collagen and smooth muscle cell attenuation. Recent work by Rauch et al. links P2Y$_{12}$ expression with VSMC mitogenesis via a thrombin-induced pathway and may provide a potential mechanism for the effect we observed. Interestingly, following exposure to thrombin, they found P2Y$_{12}$ surface expression being significantly up-regulated and P2Y$_{12}$-induced mitogenic effects enhanced. In an inflammatory setting such as atherogenesis, where thrombin generation is likely, it is probable that P2Y$_{12}$-potentiating VSMC mitogenesis contributes to lesion progression. Indeed, P2Y$_{12}$-positive VSMCs have been identified within atherosclerotic lesions. Furthermore, to eliminate the possibility of differences in thrombin generation on the interpretation of our findings, we conducted PT and aPTT tests and found no difference in response between chimeric groups.

The smaller lesions observed in these vessel wall P2Y$_{12}$-deficient mice suggest that VSMC P2Y$_{12}$ expression does influence lesion size, perhaps via VSMC mitogenesis, however this is yet to be explored. Absence of smooth muscle actin expression within these lesions, compared with positive staining seen in those mice which do express vessel wall P2Y$_{12}$, lends some support to this hypothesis, although whether lesion progression is delayed or altered in the absence of VSMC P2Y$_{12}$ remains to be seen. Indeed, little is known of the true effects of P2Y$_{12}$ deficiency on VSMC development, phenotype or plasticity and so extensive investigation is required to find the exact mechanism behind these findings.

Clopidogrel is widely used in the treatment of acute coronary syndromes; however, next-generation agents, such as ticagrelor, are increasingly employed due to their superior inhibitory effects and greater clinical efficacy. Although particularly effective at preventing thrombosis, evidence of their effects on atherogenesis is limited. Studies investigating clopidogrel treatment on atherosclerosis offer conflicting results and so we were interested to explore whether P2Y$_{12}$ inhibitors were able to inhibit this vessel wall effect on atherogenesis. We found that, despite effective platelet P2Y$_{12}$ inhibition, neither ticagrelor nor clopidogrel had any effect on early atherogenesis in ApoE$^{-/-}$ mice. Studies have shown that ticagrelor and another reversibly binding P2Y$_{12}$ inhibitor cangrelor are capable of inhibiting VSMC contraction in denuded arteries, but clopidogrel had no effect with the short half-life of the active metabolite being postulated as a potential contributory factor to this lack of response. Given our findings we deduce that, at doses which yield high levels of platelet P2Y$_{12}$ inhibition, ticagrelor and clopidogrel are unable to inhibit VSMC P2Y$_{12}$-mediated effects on atherogenesis. Nonetheless, further investigation is needed to elucidate the action of these agents on VSMC P2Y$_{12}$ receptors in vivo and deduce their ability to penetrate the endothelium.

Ticagrelor has been shown to block adenosine re-uptake by erythrocytes and so may have other, non-P2Y$_{12}$-mediated, vessel wall effects. Adenosine is known to induce nitric oxide production in both VSMC and endothelial cells, which suppresses adhesion molecule expression such as VCAM-1 and also inhibits VSMC proliferation. Furthermore, clinical studies show that ticagrelor offers a reduced mortality risk, compared with clopidogrel, in patients with acute coronary syndromes but the mechanisms for this mortality reduction remain to be established. Given ticagrelor’s potential athero-protective activity, we investigated non-P2Y$_{12}$-mediated effects on atherogenesis using DK mice. Neither ticagrelor nor clopidogrel treatment had any effect on lesion development, indicating that they have no off-target effects that may influence early atherogenesis. However, consideration must also be made for the possible effects of P2Y$_{12}$ deficiency on VSMC development and behaviour in this model, which may negate any effects on the vessel wall.

In conclusion, these results demonstrate that platelet P2Y$_{12}$ plays no role in early atherogenesis despite its dominant role in platelet reactivity and, similarly, the platelet P2Y$_{12}$ inhibitors ticagrelor and clopidogrel have no effect at this stage of disease. This is in contrast to published data that document the involvement of platelet P2Y$_{12}$ in later atherogenesis and its thrombotic complications. On the other hand, we have demonstrated an important role for vessel wall P2Y$_{12}$ in early atherogenesis, particularly in those regions of the vasculature which are associated with turbulent blood flow. This work emphasizes the complexity of cellular roles within atherogenesis and how the nature and impact of these cell populations may change as disease evolves. Further investigation is necessary to clarify the extent to which vessel wall and platelet P2Y$_{12}$ influence atherogenesis throughout disease progression to provide a clearer understanding of potential pharmacological targets in the prevention and management of atherosclerosis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Vessel wall P2Y12 potentiates early atherogenesis

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