Reduced Atherosclerotic Lesions in P2Y<sub>1</sub>/Apolipoprotein E Double-Knockout Mice

The Contribution of Non–Hematopoietic-Derived P2Y<sub>1</sub> Receptors

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Background—The P2Y<sub>1</sub> receptor plays a key role in arterial thrombosis and is widely expressed in many cell types involved in atherosclerosis. The aim of this study was to evaluate its potential involvement in the development of atherosclerotic lesions.

Methods and Results—Apolipoprotein E–deficient (ApoE<sup>−/−</sup>) and P2Y<sub>1</sub><sup>−/−</sup>/ApoE<sup>−/−</sup> mice were maintained on regular chow for 17 or 30 weeks before analysis of atherosclerotic lesions. At 17 weeks, lesions in the aortic sinus and entire aorta were smaller in P2Y<sub>1</sub><sup>−/−</sup>/ApoE<sup>−/−</sup> compared with those in ApoE<sup>−/−</sup> animals. At 30 weeks, the aortic sinus lesions in P2Y<sub>1</sub><sup>−/−</sup>/ApoE<sup>−/−</sup> mice were still diminished in size and displayed reduced inflammation, reflected by decreased macrophage infiltration and diminished VCAM-1 immunostaining, compared with those in ApoE<sup>−/−</sup> mice. They also had a lower smooth muscle cell content. Unexpectedly, bone marrow transplantation showed that the absence of the P2Y<sub>1</sub> receptor in blood cells only led to no significant modification of the lesion compared with control ApoE<sup>−/−</sup> reconstituted animals. Conversely, the absence of the P2Y<sub>1</sub> receptor except in blood cells resulted in a reduction in lesion size similar to that in control P2Y<sub>1</sub><sup>−/−</sup>/ApoE<sup>−/−</sup> reconstituted mice, pointing to a role of non–hematopoietic-derived P2Y<sub>1</sub> receptors, most likely the endothelial or smooth muscle cell P2Y<sub>1</sub> receptors. In addition, although this was not statistically significant, plasma cholesterol levels were consistently decreased in P2Y<sub>1</sub><sup>−/−</sup> animals, suggesting that a modification of lipid metabolism could be responsible for the observed phenotype.

Conclusion—The P2Y<sub>1</sub> receptor contributes to atherosclerosis, primarily through its role in non–hematopoietic-derived cells. (Circulation. 2008;118:754-763.)

Key Words: atherosclerosis ■ blood cells ■ immunohistochemistry ■ plaque ■ transplantation

In blood vessels, extracellular nucleotides are involved in a number of physiological functions such as vascular tone, hemostasis, and the inflammatory response because of their direct action on smooth muscle cells, endothelial cells, leukocytes, and platelets. In addition to these short-term effects, nucleotides also are implicated in long-term trophic effects on cell growth, proliferation, and death. Because all of these events have important implications for atherosclerosis, extracellular nucleotides and their receptors could play a role in the progression of this disease. Nucleotides act through specific receptors belonging to the P2 family, which consists of 2 classes of membrane receptor: ligand-gated P2X cation channels and G protein–coupled P2Y receptors. To date, 7 subtypes of mammalian P2X receptors and 8 subtypes of P2Y receptors have been cloned and characterized. However, their role in the development of atherosclerosis has been poorly investigated. The only published study was carried out in collared rabbit carotid arteries and concerned the involvement of the P2Y<sub>2</sub> receptor in the development of intimal hyperplasia, a phenomenon occurring in both atherosclerosis and restenosis after percutaneous transluminal angioplasty.

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The P2Y<sub>1</sub> receptor is widely distributed in many tissues of the body and is present in vascular endothelial cells and circulating blood cells such as leukocytes and platelets. Its role in platelet functions has been extensively investigated. This receptor is necessary for normal platelet aggregation in response to ADP and contributes to interactions between platelets and leukocytes and to the exposure of tissue factor on activated leukocytes. The P2Y<sub>1</sub> receptor plays a key role in arterial thrombosis, as has been demonstrated with P2Y<sub>1</sub>-deficient (P2Y<sub>1</sub><sup>−/−</sup>) mice and P2Y<sub>1</sub> antagonists in various models of experimental thrombosis. Hence, this receptor could represent an interesting target for new antiplatelet compounds. The endothelial P2Y<sub>1</sub> receptor contributes, like other P2 receptor subtypes, to nucleotide-induced
relaxation\textsuperscript{16,17} and was recently shown also to be involved in human endothelial cell migration.\textsuperscript{18} The role of P2Y\textsubscript{1} in leukocytes is less well established.

It has been known for a very long time that blood platelets not only have important functions in hemostasis and thrombus formation but are also involved in the development of atherosclerosis.\textsuperscript{19–21} More recent reports have provided insight into the molecular mechanisms involved in these processes, notably the part played by platelet activation and adhesion to the vessel wall.\textsuperscript{22–25} In addition, the release from platelets of a variety of proinflammatory cytokines, including interleukin-1β and RANTES,\textsuperscript{26} and exposure of CD40L\textsuperscript{27} trigger endothelial inflammation,\textsuperscript{27–29} whereas exposure of P-selectin on the surface of platelets initiates their direct interaction with leukocytes, thereby regulating leukocyte function and overall leading to the recruitment of monocytes to the vessel wall.

Thus, because the P2Y\textsubscript{1} receptor plays an important role in platelet physiology and is present in all cell types and tissues involved in inflammation and atherosclerosis, we wished to explore its potential involvement in the development of atherosclerosis. We therefore crossed apolipoprotein-deficient (ApoE\textsuperscript{-/-}) mice with P2Y\textsubscript{1} migrators (P2Y\textsubscript{1} migrators) to generate P2Y\textsubscript{1}/ApoE double-knockout mice (P2Y\textsubscript{1}/ApoE\textsuperscript{-/-}). These animals were maintained on regular chow before collection of the whole aortas and hearts for analysis of the size and composition of atherosclerotic lesions. The latter were found to be reduced in size and to display decreased macrophase infiltration and smooth muscle cell proliferation in P2Y\textsubscript{1}/ApoE\textsuperscript{-/-} compared with ApoE\textsuperscript{-/-} mice. Bone marrow transplantation was performed to determine the relative contributions to the development of atherosclerosis of the P2Y\textsubscript{1} receptors expressed on blood cells and in the rest of the body. Unexpectedly, the results exclude the involvement of platelet and other blood cell P2Y\textsubscript{1} receptors and point to a role of the P2Y\textsubscript{1} receptors of other tissues, most likely the vasculature and/or liver. In view of the known involvement of this receptor in platelet physiology and thrombosis, these findings might be of importance for the development of new antiatherothrombotic strategies.

**Methods**

**Mice, Genotype, and Diet**

P2Y\textsubscript{1}/ApoE\textsuperscript{-/-} mice were generated by mating ApoE\textsuperscript{-/-} mice (Charles River, L’Arbresle, France) with P2Y\textsubscript{1} migrators. After genotyping by polymerase chain reaction analysis, littermates from the F2 generation of this crossbreed were used to establish P2Y\textsubscript{1}/ApoE\textsuperscript{-/-} mating, and the resulting progeny was used in our study. All animals were of pure (9-generation backcross) C57BL/6 genetic background and were maintained in the animal facilities of the Etablissement Français du Sang-Alsace. The mice were fed standard mouse chow containing 3% fat (Charles River) from weaning until 17 or 30 weeks of age and then were killed for analysis of atherosclerotic lesions. Ethics approval for animal experiments was obtained from the French Ministry of Research in accordance with the European Union guidelines.

**Plasma Cholesterol and Triglyceride Analysis**

At the age of 17 or 30 weeks, blood was drawn from the abdominal aorta of anesthetized mice into citrate (3.15%) anticoagulant. Plasma was isolated by centrifugation at 10,000g for 10 minutes and maintained at 4°C. Concentrations of total cholesterol and triglycerides were determined in total plasma with an enzymatic assay according to the manufacturer’s instructions (Cholesterol SL and Triglycerides Mono SL New, Elitech Diagnostics, Sees, France).

**Quantification of the Size of the Aortic Sinus Lesions**

The aorta was perfused with 2 mL of 4% paraformaldehyde and placed in 4% paraformaldehyde overnight. The fixed hearts were washed in PBS, embedded in paraffin, and cross-sectioned. The sections were then collected until reaching the junction of the heart muscle and aorta, where the valve cups become visible. Three consecutive 5-μm sections were then collected for each slide, and 20 slides were prepared from each animal. Five sections at 60-μm intervals were stained with elastica van Gieson reagent for lesion area measurement or Sirius Red for detection of collagen fibers. The lesion area was measured with image analysis (MetaMorph software, Universal Imaging Corp, Downingtown, Pa) by an operator blinded to genotype. Values reported represent the mean lesion area in 5 sections for each animal.

**Immunohistochemical Analysis of the Aortic Sinus Lesions**

The macrophage content was analyzed by immunostaining with a rat anti-mouse Mac-3 monoclonal antibody (M3/84, 550292, BD PharMingen, San Diego, Calif) that was revealed with a biotin-conjugated anti-rat antibody. Smooth muscle cells were stained with a rabbit polyclonal antibody against human smooth muscle α-actin (RB-9010-P, Interchim, Montluçon, France), followed by a biotin-conjugated anti-rabbit antibody. Antibodies were revealed with a peroxidase-linked avidin/biotin detection system (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif), and irrelevant immunoglobulins were used as negative controls. Vascular cell adhesion molecule-1 (VCAM-1) immunostaining was performed on frozen sections with a rat anti-mouse VCAM-1 monoclonal antibody (M/K-2, SouthernBiotech, Birmingham, Ala). Macrophage-, smooth muscle cell-, and VCAM-1–positive regions were quantified in 5 sections at 60-μm intervals for each animal by measuring the area staining positive for the respective marker with MetaMorph software. The percentage area of a lesion positive for α-actin, Mac-3, or VCAM-1 was calculated as the α-actin–, Mac-3–, or VCAM-1–positive area of the lesion divided by its total area.

**Quantification of the Lesion Size in the Entire Aorta**

The aortas of anesthetized mice (17 or 30 weeks old) were excised between the subclavian branch and the iliac bifurcation. The cleaned aortas were cut open longitudinally, fixed in 60% isopropanol for 2 minutes, and stained with Oil Red O. The total and atherosclerotic areas of each aorta were measured with MetaMorph software, and the ratio of the atherosclerotic area to the total area was calculated.

**Bone Marrow Transplantation**

Bone marrow was harvested by flushing the femurs of the donor mice with Hanks’ balanced salt solution under sterile conditions, and the cells were washed and re suspended in that solution. Recipient 5-week-old male mice were sublethally irradiated (12 Gy) with an electron accelerator (Aeria, Illkirch, France). Three hours after irradiation, the recipient mice received 3×10\textsuperscript{6} freshly prepared sterile donor bone marrow cells by tail vein injection. During the first 6 weeks after transplantation, the mice were kept in a sterile unit and given sterile food and water, after which they were maintained under the regular conditions of the animal facilities until 30 weeks of age.

**Control of Bone Marrow Reconstitution**

Because the P2Y\textsubscript{1} receptor is absolutely necessary for platelet aggregation in response to ADP, the successful reconstitution of bone marrow–derived cells was verified in each mouse by measuring the aggregation response of washed platelets to ADP.\textsuperscript{30} Aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Canada).
Blood cell counts were determined in EDTA-anticoagulated blood with a Scil Vet ABC automatic cell counter (Scil Animal Care Company, Holtzheim, France) set to murine parameters.

Statistical Analyses
Statistical analyses were performed with GraphPad software (Prism 3.0). Data are reported as mean ± SEM and were compared by use of Student’s t test to evaluate 2-tailed levels of significance. In the case of bone marrow transplantation experiments, data were compared by use of 1-way ANOVA, followed by a Bonferroni correction posthoc test. Results were considered to be significant at values of \( P < 0.05 \).

Results
P2Y1 Receptor Deficiency Results in a Reduction of Atherosclerosis in ApoE\(^{-/-}\) Mice
Analysis of the aortic preparations from 17-week-old animals stained with Oil Red O revealed a statistically significant

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Atherosclerotic lesion size in 17-week-old ApoE\(^{-/-}\) and P2Y1\(^{-/-}\) mice. Mice were maintained on a standard low-fat mouse chow diet for 17 weeks. A, Surface coverage of the entire aorta with atherosclerotic lesions. Left, Representative photomicrographs of aortas collected between the subclavian and iliac branches and stained with Oil Red O (lipid staining in red). Right, Quantitative computer-assisted image analysis of lipid deposition in the aorta. Data represent the percentage surface area of the aorta occupied by lesions in P2Y1\(^{-/-}\)/ApoE\(^{-/-}\) and ApoE\(^{-/-}\) mice (12.4 ± 1.2% versus 21.2 ± 3.3%; \( n = 6 \); \( *P = 0.0301 \), Student’s t test). B, Atherosclerotic lesion size in the aortic sinus. Top, Representative photographs of a cross section of the aortic sinus stained with elastica van Gieson reagent in ApoE\(^{-/-}\) and P2Y1\(^{-/-}\)/ApoE\(^{-/-}\) mice. Bottom, Quantitative computer-assisted image analysis of lesions in the aortic sinus. Data represent the mean ± SEM lesion area in 5 sections for each animal. The lesion area was reduced by 31% in the aortic sinus of P2Y1\(^{-/-}\)/ApoE\(^{-/-}\) vs ApoE\(^{-/-}\) mice (0.061 ± 0.01 mm\(^2 \) [\( n = 11 \)] vs 0.089 ± 0.01 mm\(^2 \) [\( n = 14 \)]; \( P = 0.0994 \)). Scale bar = 250 μm.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
43% reduction in the surface covered by atherosclerotic lesions in $P2Y_1^{-/-}/ApoE^{-/-}$ compared with $ApoE^{-/-}$ mice (12.4±1.2% versus 21.2±3.3%; n=6; *P=0.0301; Figure 1A). In the aortic sinus, the elastica van Gieson–stained lesion area was 31% less in $P2Y_1^{-/-}/ApoE^{-/-}$ compared with $ApoE^{-/-}$ mice (0.061±0.01 mm² [n=11] versus 0.089±0.01 mm² [n=14]; P=0.0994; Figure 1B).

With increasing age (30 weeks), the lesions had spread throughout the entire aorta. The percentage of surface area occupied by the lesions still tended to be reduced in $P2Y_1^{-/-}/ApoE^{-/-}$ compared with $ApoE^{-/-}$ animals, although the difference did not reach statistical significance (22.6±2.1% [n=15] versus 25.9±1.7% [n=16]; P=0.2447; Figure 2A). At this time point, the lesions in the aortic sinus became
larger and more complicated in both ApoE−/− and P2Y1−/−/ApoE−/− mice. However, a highly significant difference between the two genotypes was observed, with a 24% smaller aortic sinus lesion area in P2Y1−/−/ApoE−/− mice (0.41±0.03 mm² [n=15] versus 0.54±0.03 mm² [n=16]; **P=0.003; Figure 2B).

**Effect of P2Y1 Receptor Deficiency on the Composition of Aortic Sinus Lesions**

Immunohistochemical analysis of the aortic sinus was performed to determine the composition of the lesions. At 17 weeks, both ApoE−/− and P2Y1−/−/ApoE−/− mice had developed fatty streaks containing mostly macrophages but no smooth muscle cells as evidenced by the absence of α-actin staining (data not shown). At the age of 30 weeks, the lesions had progressed to the stage of fibrous plaques in both genotypes. However, Mac-3 staining revealed a marked reduction in the absolute surface area occupied by macrophages in the lesions of P2Y1−/−/ApoE−/− mice compared with those of ApoE−/− mice (0.097±0.008 mm² [n=15] versus 0.160±0.014 mm² [n=16]; **P=0.0019; Figure 3A and 3B).

The percentage of total plaque area occupied by macrophages, expressed as the percentage of the cross-sectional area of a lesion, also was significantly reduced in P2Y1−/−/ApoE−/− compared with ApoE−/− mice (23±1% [n=15] versus 29±2% [n=16]; *P=0.0104; Figure 3B), as was the percentage area of VCAM-1 immunostaining (9±3% [n=6] versus 22±3% [n=9]; *P=0.0139; Figure 3A and 3B). The smaller atherosclerotic plaques of P2Y1−/−/ApoE−/− mice further presented a significantly reduced accumulation of smooth muscle cells (0.015±0.004 mm² [n=15] versus 0.035±0.006 mm² [n=14]; *P=0.0113; Figure 3A and 3B), as revealed by α-actin staining. When results were normalized to the total lesion area, the percentage covered by smooth muscle cells was again significantly lower in P2Y1−/−/ApoE−/− compared with ApoE−/− animals (3±1% [n=15] versus 8±1% [n=15]; *P=0.003; Figure 3A and 3B).
versus 7±1% [n=14]; **P=0.0376; Figure 3B). In contrast, staining with Sirius Red showed the lesions of P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> mice to contain comparable proportions of collagen fibers (52±2% [n=15] versus 50±3% [n=16]; P=0.6597; Figure 3A and 3B), indicating that P2Y1 receptor deficiency did not modify the collagen content of the lesions.

**Analysis of Atherosclerotic Lesions in 30-Week-Old Mice Having Undergone Bone Marrow Transplantation**

Bone marrow transplantation was used to determine the relative contributions of the P2Y1 receptors of blood cells (platelets, monocytes/macrophages, lymphocytes) and P2Y1 receptors expressed in other tissues to the development of atherosclerosis. At the age of 5 weeks, male ApoE<sup>−/−</sup> and P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> mice were sublethally irradiated and transplanted with bone marrow of either genotype to give 4 groups of animals: (1) ApoE<sup>−/−</sup> recipient mice transplanted with ApoE<sup>−/−</sup> bone marrow (wt-wt) or (2) P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> bone marrow (ko-wt) and (3) P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> recipient mice transplanted with ApoE<sup>−/−</sup> bone marrow (wt-ko) or (4) P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> bone marrow (ko-ko). Thus, the nomenclature of the chimeras denotes first the P2Y1 genotype of the hematopoietic-derived cells and second that of the rest of the body.

Bone marrow reconstitution was controlled in each recipient mouse by measuring the aggregation of washed platelets in response to ADP. As expected, ADP (10 μmol/L) induced the aggregation of platelets derived from wt-wt or wt-ko mice but not of platelets derived from ko-wt or ko-ko mice (Figure 4), demonstrating efficient bone marrow transplantation and reconstitution in the recipient animals.

At the age of 30 weeks, the absence of the P2Y1 receptor in control reconstituted ko-ko mice led to a 27% smaller aortic sinus lesion area than in control reconstituted wt-wt mice (0.38±0.05 mm<sup>2</sup> [n=8] versus 0.52±0.04 mm<sup>2</sup> [n=11]; **P<0.05; Figure 5), consistent with the results obtained in nontransplanted ApoE<sup>−/−</sup> and P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> animals (Figure 2A). Unexpectedly, the absence of the P2Y1 receptor in blood cells only (ko-wt mice) did not result in any reduction in lesion size compared with wt-wt mice (0.58±0.03 mm<sup>2</sup> [n=7] versus 0.52±0.04 mm<sup>2</sup> [n=11]; P>0.05), suggesting that the blood cell P2Y1 receptors do not contribute to the development of atherosclerotic lesions in mice. In contrast, when the P2Y1 receptor was absent in all cell types and tissues except blood cells (wt-ko mice), the lesion area was strongly decreased, similar to ko-ko mice (0.43±0.04 mm<sup>2</sup> [n=8] versus 0.38±0.05 mm<sup>2</sup> [n=8]; P>0.05; Figure 5). Consistent with these results, the difference in atherosclerotic lesion size between wt-ko and ko-ko mice was highly significant (0.43±0.04 mm<sup>2</sup> in wt-ko mice [n=8] versus 0.58±0.03 mm<sup>2</sup> in ko-ko mice [n=8]; **P<0.05). Altogether, these data indicate that the contribution of the P2Y1 receptor to atherosclerosis depends on its expression in tissues other than blood.

Immunohistochemical staining for macrophages revealed a marked reduction in the absolute surface area occupied by macrophages in the lesions of ko-ko mice compared with those of wt-wt mice (0.10±0.01 mm<sup>2</sup> [n=8] versus 0.18±0.02 mm<sup>2</sup> [n=11]; **P<0.01), confirming the results obtained in nontransplanted ApoE<sup>−/−</sup> and P2Y1<sup>−/−</sup>/ApoE<sup>−/−</sup> animals. Consistent with this, the area staining for macrophages was not significantly different in the lesions of ko-wt mice compared with those of wt-wt mice or in the lesions of ko-wt mice compared with those of ko-ko mice. However, the difference between ko-wt and wt-ko, although measurable, did not reach statistical significance (data not shown).

The smooth muscle cell content also was reduced in the lesions of ko-ko mice compared with those of wt-wt mice,
although the difference did not reach statistical significance, probably because of the smaller number of animals in this experiment (0.019±0.007 versus 0.038±0.008 mm² [n=8]; P>0.05).

Body Weight and Plasma Lipid Measurements

Body weights were comparable in all animals used in the experiments. The plasma lipid profile of nontransplanted mice displayed a nonsignificant reduction of 10% in total cholesterol in P2Y1−/−/ApoE−/− compared with ApoE−/− mice. Interestingly, in transplanted mice lacking the P2Y1 receptor in all tissues except blood, total cholesterol was clearly decreased (15% to 20%) compared with levels in animals expressing the P2Y1 receptor throughout the body, independently of its presence or absence in blood cells (Tables 1 and 2). Although this difference was not statistically significant, probably because the number of animals was too small, it might be relevant to the contribution to atherosclerosis. In contrast, the triglyceride levels were not affected.

Discussion

We report here that the P2Y1 receptor plays a role in both early and late stages of the development of atherosclerotic lesions. At 17 weeks of age, the lesions formed in the aortic
Table 1. Characteristics of ApoE−/− and P2Y1−/−/Apoe−/− Mice Fed a Chow Diet for 17 or 30 Weeks

<table>
<thead>
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<th>ApoE−/−</th>
<th>P2Y1−/−/Apoe−/−</th>
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<td>17-week-old mice, n</td>
<td>14</td>
<td>11</td>
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<tr>
<td>Weight, g</td>
<td>31.9±0.7</td>
<td>33.3±0.9</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>442±24</td>
<td>392±28</td>
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<td>Triglycerides, mg/dL</td>
<td>63±4</td>
<td>107±17</td>
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<td>30-week-old mice, n</td>
<td>16</td>
<td>15</td>
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<tr>
<td>Weight, g</td>
<td>36.8±0.7</td>
<td>35.0±0.7</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<td>463±30</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>72±7</td>
<td>85±6</td>
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The atherosclerotic lesions of P2Y1−/−/Apoe−/− mice were smaller than those in ApoE−/− mice. At the age of 30 weeks, the aortic root lesions of P2Y1−/−/Apoe−/− mice were still reduced in size and displayed less inflammation, reflected by decreased macrophage infiltration and diminished VCAM-1 immunostaining, compared with the lesions of ApoE−/− animals. There was also a reduction in the smooth muscle cell content, whereas the collagen content remained similar between the 2 genotypes.

The P2Y1 receptor is widely expressed in most cell types and tissues of the body; several, including at least blood cells, the vasculature, and the liver, may be involved in atherosclerosis. Our bone marrow transplantation experiments indicate that blood cell P2Y1 receptors are not involved in the development of atherosclerosis. This is somewhat surprising because the platelet P2Y1 receptor is known to play a role in platelet activation, P-selectin exposure, platelet-leukocyte interactions, and expression of tissue factor on monocytes. On the other hand, activated platelets contribute substantially to atherosclerosis. One would therefore have expected the platelet P2Y1 receptor to play a more important role here. For instance, ticlopidine, an irreversible inhibitor of the second platelet ADP receptor, the P2Y12 receptor, has been found to inhibit atherosclerotic plaque progression. However, using the analogous P2Y12 inhibitor clopidogrel, another group did not confirm these results. In any case, it seems unlikely that the platelet P2Y1 receptor could be responsible for the phenotype of our P2Y1-deficient mice. At least 2 other possibilities, not mutually exclusive, might explain the results: the role of the P2Y1 receptor in the vasculature, in endothelial or smooth muscle cells, and an effect of the P2Y1 deficiency on metabolic pathways involved in atherosclerosis.

The expression of the P2Y1 receptor in endothelial cells is well established, whereas its presence in the underlying smooth muscle cells is uncertain. This receptor has been shown to contribute to endothelium-dependent relaxation through nitric oxide and prostacyclin release. However, the function of the P2Y1 receptor in endothelial cells is largely unknown. Several findings indicate that adenine nucleotides may contribute to an inflammatory state of the endothelium. Thus, these molecules have been reported to increase the expression of E-selectin on primary porcine or bovine aortic endothelial cells; to upregulate the secretion and expression of interleukin-6, interleukin-8, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1 in dermal endothelial cells; and to increase the adhesion of leukocytes to endothelial cells. It remains to be determined whether the endothelial P2Y1 receptor could contribute to these phenomena. This would suggest that P2Y1 displays a dual role, contributing to the antithrombogenic properties of endothelium by its role on nitric oxide and prostaglandin E2 release under physiological conditions but also to endothelial cell activation under pathological conditions. Such a dual role has already been reported for another P2Y receptor subtype, P2Y2. This receptor contributes to endothelium-dependent relaxation and to the expression of VCAM-1, which mediates the adhesion of monocytes to vascular endothelium.

The atherosclerotic lesions of P2Y1−/−/Apoe−/− mice also contained fewer smooth muscle cells than those of ApoE−/− mice, indicating that P2Y1 receptor deficiency might influence the behavior of these cells. This could be an indirect consequence of the reduced monocyte infiltration of the lesions and resultant decreased secretion of chemokines able to attract smooth muscle cells or stimulate their proliferation. On the other hand, the P2Y1 receptor could be directly involved in the proliferation of smooth muscle cells because extracellular ATP and ADP can act as mitogens for vascular smooth muscle cells, alone or in synergy with growth factors like platelet-derived growth factor, whereas upregulation of P2Y1 receptor expression has been observed in vascular smooth muscle cells in culture during the change from a contractile to a synthetic phenotype.

Concerning a possible role of the P2Y1 receptor in metabolic pathways involved in atherosclerosis and notably in lipid metabolism, very few studies have been reported to date. However, one must take into consideration our results for blood cholesterol concentrations, which, although not statistically significant, consistently indicated lower lipidemia in P2Y1−/−/Apoe−/− mice than those in ApoE−/− animals. At the age of 30 weeks, the aortic root lesions of P2Y1−/−/Apoe−/− mice were still reduced in size and displayed less inflammation, reflected by decreased macrophage infiltration and diminished VCAM-1 immunostaining, compared with the lesions of ApoE−/− animals. There was also a reduction in the smooth muscle cell content, whereas the collagen content remained similar between the 2 genotypes.

Table 2. Characteristics of 30-Week-Old Mice That Received Bone Marrow Transplants

<table>
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<th>ko-wt</th>
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<td>Weight, g</td>
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<td>27.3±0.7</td>
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<td>Total cholesterol, mg/dL</td>
<td>373±26</td>
<td>343±51</td>
<td>298±29</td>
<td>301±36</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>80±11</td>
<td>52±7</td>
<td>88±8</td>
<td>110±20</td>
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Our results may have important clinical implications. Indeed, the P2Y1 receptor plays a key role in platelet activation and in arterial thrombosis, as has been evidenced in P2Y1-deficient mice and using selective P2Y1 antagonists in vitro in platelet function studies and in vivo in animal models of thrombosis. It is thus a potential promising target for new antiplatelet drugs. The demonstration that this receptor also is involved in atherosclerosis obviously adds interest in targeting simultaneously 2 separate aspects of atherothrombosis, i.e., platelet activation and development of atherosclerosis. Moreover, because the P2Y1 receptor plays a more minor role in normal hemostasis compared with the P2Y12 receptor, one can expect a smaller risk of bleeding with P2Y1-targeting...
drugs, which is the major limitation of aggressive antiplatelet therapy, especially when targeting the P2Y12 receptor. P2Y1-targeting drugs might thus be efficient on a long-term basis in patients requiring long-term treatment.

Acknowledgment

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

The P2Y1 receptor plays a key role in platelet activation and arterial thrombosis, as has been evidenced in P2Y1-deficient mice and through the use of selective P2Y1 antagonists in vitro in platelet function studies and in vivo in animal models of thrombosis. It is thus a potentially promising target for new antiplatelet drugs. The demonstration that this receptor also is involved in atherosclerosis obviously adds interest in targeting simultaneously 2 separate aspects of atherothrombosis, ie, platelet activation and development of atherosclerosis. Moreover, because the P2Y1 receptor plays a more minor role in normal hemostasis compared with the P2Y12 receptor, one can expect a smaller risk of bleeding with P2Y1-targeting drugs, which is the major limitation of aggressive antiplatelet therapy, especially when targeting the P2Y12 receptor. P2Y1-targeting drugs might therefore be efficient on a long-term basis in patients requiring chronic treatment.
Reduced Atherosclerotic Lesions in P2Y1/Apolipoprotein E Double-Knockout Mice: The Contribution of Non–Hematopoietic-Derived P2Y1 Receptors
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