Roles of thromboxane A$_2$ and prostacyclin in the development of atherosclerosis in apoE-deficient mice


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Production of thromboxane (TX) A$_2$ and PG I$_2$/prostacyclin (PGI$_2$) is increased in patients with atherosclerosis. However, their roles in atherogenesis have not been critically defined. To examine this issue, we cross-bred atherosclerosis-prone apoE-deficient mice with mice deficient in either the TXA receptor (TP) or the PGI receptor (IP). Although they showed levels of serum cholesterol and triglyceride similar to those of apoE-deficient mice, apoE$^{-/-}$TP$^{-/-}$ mice exhibited a significant delay in atherogenesis, and apoE$^{-/-}$IP$^{-/-}$ mice exhibited a significant acceleration in atherogenesis compared with mice deficient in apoE alone. The plaques in apoE$^{-/-}$TP$^{-/-}$ mice showed partial endothelial disruption and exhibited enhanced expression of ICAM-1 and decreased expression of E-selectin in platelets of apoE$^{-/-}$IP$^{-/-}$ mice. Platelet activation with thrombin ex vivo revealed higher and lower sensitivity for surface P-selectin expression in platelets of apoE$^{-/-}$IP$^{-/-}$ and apoE$^{-/-}$TP$^{-/-}$ mice, respectively, than in those of apoE$^{-/-}$ mice. Intravital microscopy of the common carotid artery revealed a significantly greater number of leukocytes rolling on the vessel walls in apoE$^{-/-}$IP$^{-/-}$ mice than in either apoE$^{-/-}$TP$^{-/-}$ or apoE$^{-/-}$ mice. We conclude that TXA$_2$ promotes and PGI$_2$ prevents the initiation and progression of atherogenesis through control of platelet activation and leukocyte-endothelial cell interaction.

Introduction

It is now understood that atherosclerosis is an inflammation in the intima of large arteries that is triggered by high serum cholesterol and in which various types of cells including monocytes/macrophages, endothelial cells (ECs), smooth muscle cells (SMCs), T cells, and blood platelets exert a complex array of interaction (1). A variety of substances including cytokines, chemokines, and growth factors are suggested to induce, amplify, and modify this inflammatory process. One group of these mediators is prostanoids, which are produced from arachidonic acid by the action of COX and include various types of PGs and thromboxane (TX). Involvement of prostanoids in acute inflammation has been well documented based on the finding that aspirin-like NSAIDs are COX inhibitors. Among prostanoids, PG I$_2$/prostacyclin and TXA$_2$ have attracted particular attention for their importance in cardiovascular diseases: the former, generated by vascular ECs, is a potent platelet inhibitor and vasoconstrictor, and the latter, released from activated platelets, is a potent vasoconstrictor and platelet-aggregating agent. Indeed, low-dose aspirin that preferentially inhibits platelet-derived TXA$_2$ over endothelium-derived PGI$_2$ has been used as anti-platelet therapy for the prevention of myocardial infarction and recurrence of strokes. Although biosynthesis of PGI$_2$ and TXA$_2$ is increased in patients with atherosclerosis (2, 3), the roles of these molecules in the initiation and progression of atherosclerosis have not yet been critically examined.

The role played by prostanoids in atherogenesis has been studied mostly by examining the effects of various drugs in mice deficient in either apoE (apoE$^{-/-}$ mice) (4, 5) or the LDL receptor (LDLR) (LDLR$^{-/-}$ mice) (6). One group of such pharmacological studies has used low doses of aspirin in atherosclerotic model animals to evaluate the contribution of TXA$_2$. Cyrus et al. (7) fed LDLR$^{-/-}$ mice a high-fat diet, treated the mice with low-dose aspirin, and found that this dose of aspirin significantly retarded the development of atherosclerotic lesions, with a 64% reduction in an en face analysis and 29% in a cross-sectional analysis. In contrast, Cayatte et al. (8) used aspirin in apoE$^{-/-}$ mice and did not find retardation in the development of atherosclerotic lesions. This discrepancy could be attributed to the higher dose of aspirin used in the latter study, which may have inhibited prostanooid production in cells other than platelets, particularly PGI$_2$ in blood vessels. In the same study, Cayatte et al. (8) administered a TXA receptor (TP) antagonist, S-18886, to apoE$^{-/-}$ mice. They reported that administration of this drug indeed suppressed the extent of atherogenesis, but only marginally, by 21%. The other group of pharmacological studies has used isoform-specific COX inhibitors and has evaluated the roles of prostanoids in atherogenesis. COX exists as two isoforms encoded by two distinct genes (9): COX-1 is constitut-
tively expressed in most tissues and mediates basal physiological functions, while COX-2 is induced by various types of stimuli and works “on demand” in such conditions as inflammation. There is now substantial evidence that the majority of PGI$_2$ is produced by COX-2 in vascular ECs, whereas production of TXA$_2$ by platelets is catalyzed by COX-1 (10). The COX-2–catalyzed PGI$_2$ production probably reflects induction of COX-2 by hemodynamic shear stress in the vasculature (11). The issue of whether COX-2–derived PGI$_2$ exerts any protective effect on atherosclerosis is important, given that many juvenile patients with arthritis are treated with selective COX-2 inhibitors (12) and a large-scale study (VIGOR) indicated an increased tendency for cardiovascular accidents associated with the use of such drugs versus the nonselective COX inhibitor naproxen (discussed in ref. 13). Experiments examining the effects of COX-2 inhibitors in atherogenesis have yielded conflicting results. One study in which an MF-tricyclic was administered to apoE–/– mice found exaggeration of atherosclerosis (14), and one study examining the effect of rofecoxib in LDLR–/– mice detected a small but significant suppression in the development of atherosclerosis (15). The former study (14) did not specify the gender of the mice studied and may be difficult to interpret. However, two other studies, one of nimesulide in LDLR–/– mice (16) and the other of SC-236 in apoE–/– mice (17), did not find significant effects. These studies, except for the study using MF-tricyclic (14), all detected similar suppression of PGI$_2$ production in animals given these drugs. However, the suppression remained partial,
supporting a view that both COX-1 and COX-2 contribute to PGII production under pathological conditions such as atherosclerosis (3). Thus, pharmacological approaches using various drugs have produced variable and inconclusive results and have failed to provide a cohesive picture on the contribution of prostanooids, including PGI\(_2\), to atherogenesis. This probably reflects the inherent limitations associated with pharmacological studies, such as differences in the potency and specificity of individual drugs and differences in the experimental protocols and animal models. Moreover, it is difficult in principle to evaluate contribution of each prostanooid by the use of COX inhibitors, because each isoform is capable of producing more than one type of prostanooid in a variety of tissues. For example, TXA\(_2\) is produced not only by COX-1 in blood platelets but also by COX-2 in macrophages, which is also believed to produce PGE\(_2\) in atheromatous plaques. The importance of COX-2 in macrophages was suggested by the reduction in atherosclerosis found in LDLR\(^{-/-}\) mice reconstituted with COX-2\(^{-/-}\) fetal liver cells (15).

In order to conquer these limitations, we have examined the development of atherosclerosis in mice deficient in prostanooid receptors for individual molecules (TXA\(_2\) and PGI\(_2\)). TXA\(_2\) and PGI\(_2\) exert their effects through interaction with cell surface receptors specific to each molecule, TP and the PGI receptor (IP), respectively (18). These receptors are encoded by distinct genes and are expressed differentially in the body. With the use of homologous recombination, we have generated mice that lack either TP or IP individually and have subjected the mice to models of various diseases to analyze the roles of TXA\(_2\) and PGI\(_2\) (19–29). In this work, we have cross-bred TP- and IP-deficient (TP\(^{-/-}\) and IP\(^{-/-}\)) mice with apoE\(^{-/-}\) mice and have analyzed the roles played by TXA\(_2\) and PGI\(_2\) in atherosclerotic lesion development.

### Results

**Generation and lipid profile of apoE\(^{-/-}\)TP\(^{-/-}\) and apoE\(^{-/-}\)IP\(^{-/-}\) double-KO mice.** TP\(^{-/-}\) and IP\(^{-/-}\) mice that had been backcrossed to the C57BL/6 background 10 times each were bred with apoE\(^{-/-}\) mice that had been backcrossed to the C57BL/6 background 5 times. The resultant heterozygous mice, apoE\(^{-/-}\)TP\(^{-/-}\) or apoE\(^{-/-}\)IP\(^{-/-}\) mice, were cross-bred with each other, and compound mice deficient in both apoE and TP or both apoE and IP were generated. The genes encoding IP and apoE are both located on chromosome 7, with a genetic interval of approximately 1.5 cm. To generate recombination between the genes encoding IP and apoE, we mated pairs of apoE\(^{-/-}\)IP\(^{-/-}\) double-heterozygous mice and selected offspring null either for apoE or IP (about 1% of the offspring) and cross-bred them with each other. Loss of TP or IP was assessed by PCR analysis of plasma cholesterol levels and PCR analysis. At 20 weeks of age, apoE\(^{-/-}\)TP\(^{-/-}\) and apoE\(^{-/-}\)IP\(^{-/-}\) mice showed elevated levels of both total cholesterol (TC) and total triglyceride (TG) similar to those seen in apoE\(^{-/-}\) mice (Table 1). Moreover, VLDL-cholesterol (VLDLC), LDL-cholesterol (LDLC), and HDL-cholesterol (HDLC) in apoE\(^{-/-}\)TP\(^{-/-}\) and apoE\(^{-/-}\)IP\(^{-/-}\) mice were almost identical. These findings suggest that loss of either TP or IP did not affect the hypercholesterolemia induced by apoE deficiency. apoE\(^{-/-}\)TP\(^{-/-}\) and apoE\(^{-/-}\)IP\(^{-/-}\) mice were fertile and apparently healthy. All animals were maintained on a normal chow diet and gained weight in a similar manner (data not shown).

**Atherosclerotic lesion development in apoE\(^{-/-}\)TP\(^{-/-}\) and apoE\(^{-/-}\)IP\(^{-/-}\) mice.** We used male mice of the three strains (apoE\(^{-/-}\), apoE\(^{-/-}\)TP\(^{-/-}\), and apoE\(^{-/-}\)IP\(^{-/-}\)) and examined atherosclerotic lesion development by analysis of cross-sections of the proximal aorta, en face analysis of the total aorta, and analysis of cross-sections of the innominate artery. The cross-sectional analysis of the proximal aorta was performed in the first 360 \(\mu\)m of the aortas of apoE\(^{-/-}\), apoE\(^{-/-}\)TP\(^{-/-}\), and apoE\(^{-/-}\)IP\(^{-/-}\) mice at 15, 20, and 30 weeks of age. Typical oil red O staining in each strain of mice at the respective age is shown in Figure 1C. The quantitative analysis revealed significant acceleration and delay of lesion development in apoE\(^{-/-}\)IP\(^{-/-}\) and apoE\(^{-/-}\)TP\(^{-/-}\) mice, respectively, compared with that in apoE\(^{-/-}\) mice (Figure 1D). At 15 and 20 weeks of age, the lesion areas of apoE\(^{-/-}\)IP\(^{-/-}\) mice (0.206 ± 0.016 mm\(^2\) and 0.420 ± 0.017 mm\(^2\)) were augmented significantly by 131% and 45%, respectively, compared with those of apoE\(^{-/-}\) mice (0.087 ± 0.015 mm\(^2\) and 0.290 ± 0.015 mm\(^2\)), respectively, compared with those of apoE\(^{-/-}\) mice (0.089 ± 0.015 mm\(^2\)) and apoE\(^{-/-}\)TP\(^{-/-}\) mice, respectively, compared with apoE\(^{-/-}\) mice (P < 0.05, Tukey’s t test following one-way ANOVA) (Figure 1D). After 20 weeks, lesion development in apoE\(^{-/-}\)IP\(^{-/-}\) mice appeared to quickly reach a plateau and did not show a significant difference compared with that in apoE\(^{-/-}\) mice at 30 weeks of age. In contrast, apoE\(^{-/-}\)TP\(^{-/-}\) mice showed significant delay in the lesion development; their lesion areas at 20 and 30 weeks of age (0.087 ± 0.015 mm\(^2\) and 0.183 ± 0.034 mm\(^2\)) were significantly suppressed by 70% and 58%, respectively, compared with those of apoE\(^{-/-}\) mice (0.089 ± 0.015 mm\(^2\)) and apoE\(^{-/-}\)TP\(^{-/-}\) mice, respectively, compared with apoE\(^{-/-}\) mice (P = 0.01, Tukey’s t test following one-way ANOVA) (Figure 1D).

ApoE\(^{-/-}\)IP\(^{-/-}\) and apoE\(^{-/-}\)TP\(^{-/-}\) mice showed enhancement and suppression, respectively, of atherogenesis not only locally in the aortic sinus but also globally throughout aorta. En face analysis of aortic preparations of mice at 20 weeks of age revealed significant augmentation and reduction in atherosclerotic area in apoE\(^{-/-}\)IP\(^{-/-}\) and apoE\(^{-/-}\)TP\(^{-/-}\) mice, respectively, compared with that of apoE\(^{-/-}\) mice (Figure 1E); the average lesion size in apoE\(^{-/-}\)TP\(^{-/-}\) mice (2.8% ± 0.4%) was reduced 71% compared with that in apoE\(^{-/-}\) mice (9.6% ± 0.9%), (P < 0.01, Tukey’s t test following one-way ANOVA), while that in

**Table 1**

<table>
<thead>
<tr>
<th>Mouse (mg/dl)</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>VLDLC (mg/dl)</th>
<th>LDLC (mg/dl)</th>
<th>HDLC (mg/dl)</th>
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<tr>
<td>C57BL/6 (n = 8)</td>
<td>99 ± 5</td>
<td>57 ± 4</td>
<td>6 ± 1</td>
<td>24 ± 3</td>
<td>58 ± 4</td>
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<tr>
<td>TP(^{-/-}) (n = 8)</td>
<td>85 ± 9</td>
<td>50 ± 5</td>
<td>8 ± 1</td>
<td>19 ± 3</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>IP(^{-/-}) (n = 8)</td>
<td>90 ± 6</td>
<td>55 ± 6</td>
<td>7 ± 1</td>
<td>22 ± 3</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>ApoE(^{-/-}) (n = 8)</td>
<td>535 ± 43(^A)</td>
<td>102 ± 6(^A)</td>
<td>381 ± 36(^A)</td>
<td>127 ± 5(^A)</td>
<td>27 ± 4(^B)</td>
</tr>
<tr>
<td>ApoE(^{-/-})TP(^{-/-}) (n = 8)</td>
<td>595 ± 53(^A)</td>
<td>105 ± 10(^A)</td>
<td>395 ± 60(^A)</td>
<td>138 ± 5(^A)</td>
<td>22 ± 4(^B)</td>
</tr>
<tr>
<td>ApoE(^{-/-})IP(^{-/-}) (n = 8)</td>
<td>588 ± 52(^A)</td>
<td>103 ± 8(^A)</td>
<td>430 ± 44(^A)</td>
<td>135 ± 11(^A)</td>
<td>22 ± 4(^B)</td>
</tr>
</tbody>
</table>

All data are shown as mean ± SEM. \(^A\)P < 0.01 versus C57BL/6. \(^B\)P < 0.05 versus C57BL/6. 
apoE⁻/⁻IP⁻/⁻ mice (12.3% ± 1.0%) was augmented 28% compared with that in apoE⁻/⁻ mice (P < 0.05, Tukey’s t test following one-way ANOVA) (Figure 1F). Atherosclerotic lesions in apoE⁻/⁻ mice were seen in the lesser curvature of the aortic arch and at the ostium of the brachiocephalic artery as well as in the abdominal aorta. The lesions in apoE⁻/⁻TP⁻/⁻ at this age were limited mostly to the aortic arch region, where the extent was much less. In contrast, atherosclerotic lesions in apoE⁻/⁻IP⁻/⁻ mice were more extensive than in apoE⁻/⁻ mice in every region examined (Figure 1E).

Although analysis of atherogenesis in apoE⁻/⁻ mice is carried out mostly in the aorta, atherosclerotic lesions in this strain of mice are not limited to the aorta. We noted the atherosclerotic lesion at the ostium of the brachiocephalic (innominate) artery in our en face analysis of 20-week-old mice described above. Recently, Rosenfeld et al. (30) examined the distribution of atherosclerotic lesions throughout the arterial tree of apoE⁻/⁻ mice and found a highly advanced, clinically significant lesion in the innominate artery in mice 30–60 weeks of age. We therefore examined atherosclerotic lesion development in the innominate artery by cross-sectional analysis in apoE⁻/⁻, apoE⁻/⁻TP⁻/⁻, and apoE⁻/⁻IP⁻/⁻ mice 45 weeks of age. As shown in the hematoxylin and eosin staining in Figure 1G, the lesion was found in all three strains of mice but the extent differed significantly. Whereas the plaques protruded into the arterial lumen only partially in apoE⁻/⁻ and apoE⁻/⁻TP⁻/⁻ mice, those in
apoE−/−IP−/− mice grew extensively to partially occlude the lumen. Quantitative analysis revealed significant acceleration and delay in lesion development, respectively, in apoE−/−IP−/− and apoE−/−TP−/− (right panels) mice compared with apoE−/− mice (Figure 1H).

Impaired EC integrity in atheromatous plaques of apoE−/−IP−/− mice. To investigate whether loss of TP or IP signaling had any effect on the cell composition in atheromatous plaques, we stained macrophages, SMCs, and ECs in the plaques at the aortic arches of 20-week-old mice with antibodies against the respective marker proteins. Quantification of macrophage and SMC abundance in the plaques and of endothelial integrity on the lesion surface was performed on the ten sections taken every 18 μm as described in Methods (Figure 2).

Cells staining positive for MOMA-2 were found throughout the plaques in all of apoE−/−, apoE−/−TP−/−, and apoE−/−IP−/− mice at this stage. Quantification revealed that MOMA-2–stained cells occupied about 60% of the total plaque area and there were no significant differences among the three strains (Figure 2, A and B). The fact that the area inside the plaque was stained homogenously with oil red O suggested that these MOMA-2–positive cells represent macrophage-derived foam cells and the space not occupied by macrophages represented mostly lipid cores. SMCs staining positive for α-actin accumulated and formed a layer at the top of the lesion beneath the EC layer (Figure 2A and data not shown). Quantification revealed that they comprised about 7% of the total plaque area in apoE−/− and apoE−/−TP−/− mice (Figure 2C). The proportion of SMCs in apoE−/−IP−/− mice tended to be lower than that in the other two strains of mice, but there was no significant difference. This SMC phenotype of apoE−/−IP−/− mice is at odds with the suppression
of SMC proliferation by a PG\textsubscript{I}\textsubscript{2} analog (cicaprost) in vitro (22) as well as the enhanced proliferative response found in IP-deficient mice subjected to chronic hypoxia (23) or catheter-induced carotid vascular injury (31). Impaired SMC proliferation in the absence of IP may suggest that SMC proliferation is under more complex regulation in atherosclerosis or that it may be unique to the apoE-deficient mice. These points should be clarified in future studies.

EC integrity on the plaque surface was then examined by staining of the cross-sections for two endothelial markers: von Willebrand factor (vWF) and platelet endothelial cell adhesion molecule 1 (PECAM-1). As expected, the staining was seen as a linear signal in the plaques of apoE–/– mice (85.5% ± 0.7 %) compared with that in ICAM-1 and PECAM-1 expression in ECs of apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice. To explore endothelium activation in apoE–/–TP–/– and apoE–/–IP–/– mice, we stained for ICAM-1 and PECAM-1 in ECs of apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice, we stained for ICAM-1 and PECAM-1 in ECs of all three strains of mice. In cross-sections, PECAM-1 expression was found more or less homogenously through the EC monolayer overlying the lesions, while ICAM-1 expression by endothelium was most intense at borders and the “shoulder” of the lesions in all of the three strains of mice (Figure 3A). We then performed quantitative analysis using en face confocal microscopy images (Figure 3B). In the ECs overlying the lesions, apoE–/–TP–/– mice had a significant decrease in ICAM-1 expression (53.9 ± 1.8 versus 73.1 ± 4.1; P < 0.05, Tukey’s t test following one-way ANOVA; values measured in arbitrary units based on fluorescence intensity per pixel) compared with that of apoE–/– mice, whereas ICAM-1 expression in apoE–/–IP–/– mice significantly increased (100.5 ± 7.6; P < 0.01, Tukey’s t test following one-way ANOVA) (Figure 3C). In contrast, there was no difference in ICAM-1 expression in ECs in intact areas among the three strains of mice, which was low compared with that in the atherosclerotic lesions. As for PECAM-1 expression in the atherosclerotic lesions, expression in apoE–/–TP–/– mice or apoE–/–IP–/– mice tended to increase or decrease, respectively, compared with that in apoE–/– mice, and there was a significant difference in expression between apoE–/–IP–/– and apoE–/–TP–/– mice (52.4 ± 3.2 versus 68.3 ± 1.7; P < 0.05, Tukey’s t test following one-way ANOVA) (Figure 3D). There was also no difference in PECAM-1 expression in the ECs of intact areas among the three strains of mice.

Reactivity of platelets in apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice. Because TXA\textsubscript{2} and PG\textsubscript{I}\textsubscript{2} are potent activators and suppressors, respectively, of blood platelets, chronic loss of their actions may cause alterations in platelet reactivity to an aggregating agent. Reactivity of blood platelets was therefore compared among apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice by whole-blood flow cytometry (32, 33). Briefly,
diluted blood was activated with various concentrations of thrombin and expression of P-selectin on the platelet surface was evaluated by flow cytometry. Expression of P-selectin was barely detected on unstimulated platelets, but the number of blood platelets showing surface P-selectin expression increased with increasing concentrations of thrombin. P-selectin expression was induced significantly by 0.3 U/ml thrombin and increased with 0.4 U/ml thrombin on the platelets of apoE–/– mice (Figure 4, A and B), whereas its expression was only marginally induced at 0.3 U/ml thrombin in the platelets of apoE–/–IP–/– mice (Figure 4, A and B). In contrast, an increase in the P-selectin expression was detected at doses as low as 0.1 U/ml and increased significantly with 0.2 U/ml thrombin in the platelets of apoE–/–IP–/– mice (Figure 4, A and B). These results indicate that the platelets of apoE–/–TP–/– and apoE–/–IP–/– mice have lower and higher sensitivity to thrombin, respectively, than those of apoE–/– mice.

Leukocyte adherence in the common carotid arteries of apoE–/–IP–/– mice. Finally, we examined the leukocyte-EC interaction by intravital microscopy. Rhodamine 6G was injected i.v. into apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice to label leukocytes in vivo, and interaction of the leukocytes with the wall of the common carotid artery was examined by intravital microscopy. Leukocytes rolling or adhering were seen as fluorescent dots on the wall of the artery (Figure 5A). Quantitative analysis revealed a significantly greater number of leukocytes rolling on the walls of arteries in apoE–/–IP–/– mice than in either apoE–/– or apoE–/–TP–/– mice (Figure 5B). Additionally, adhesion of leukocytes also tended to be higher in apoE–/–IP–/– mice (Figure 5C). Although rhodamine 6G also labeled platelets, we did not see thrombus formation or detachment in any strain of mice during our observation period.

Discussion

TXA₂ and PGI₂ are two major prostanoids in the cardiovascular system, being abundantly produced by blood platelets and vascular endothelium, respectively. Previous studies found that TXA₂ and PGI₂ biosynthesis is increased in patients with atherosclerosis (2, 3). In this work, we generated compound mice, apoE–/–TP–/– and apoE–/–IP–/–, and examined the roles of TXA₂ and PGI₂ in the initiation and progression of atherosclerosis. apoE–/– mice develop a spectrum of atherosclerotic lesions similar to that of humans (34). They also show elevated production of TXA₂ and PGI₂, as seen in humans (35). Thus, the apoE–/– mouse is a suitable animal model for evaluation of the roles of TXA₂ and PGI₂ in atherosclerosis. Previously, the involvement of these prostanoids in atherosclerosis was examined by the use of various COX inhibitors in this and similar animal models. However, the results obtained by those studies were variable (7, 8, 14–17). In addition, a study using a TP antagonist in apoE–/– mice showed only a marginal reduction in atherogenesis (8). In contrast to those findings in the previous studies, our study here using genetically engineered mice has demonstrated significant suppression and significant enhancement of atherosclerosis in apoE–/–TP–/– and apoE–/–IP–/– mice, respectively, suggesting strongly proatherogenic and antiatherogenic actions of TXA₂ and PGI₂, respectively. TP deficiency suppressed the extent of atherosclerosis at both 20 and 30 weeks of age. Suppression of atherosclerosis by TP deficiency is much more robust (70% at 20 weeks of age and 58% at 30 weeks of age) than that found after treatment with the TP antagonist S-18886 (about 20%) (8). We have also examined the effects of TP or IP deficiency on the development of vascular lesions in the innominate arteries of 45-week-old apoE–/– mice. Rosenfeld et al. (30) previously noted more-advanced vascular lesions in the innominate arteries in apoE–/– mice. In this study we have not only confirmed their findings in apoE–/– mice but also found that this lesion was far more advanced in apoE–/–IP–/– mice, whereas the disease progression appeared to be retarded in apoE–/–TP–/– mice.

It is noteworthy that atherogenesis was significantly accelerated and reached a plateau early in apoE–/–IP–/– mice compared with apoE–/– mice. These results indicate that signaling from PGI₂ to IP is important in preventing the initiation of atherosclerosis. Impaired PGI₂ function, moreover, appeared to affect the progression and nature of atherosclerotic plaques. Our analysis detected frequent loss of ECs in the plaques of apoE–/–IP–/– mice. In addition to ECs, we also found that the abundance of SMCs tended to be lower in the plaques of apoE–/–IP–/– mice (Figure 2). Lesions with impaired EC integrity and weaker fibrous caps are suggested to be prone to rupture (36). Recently, Cipollone and colleagues found that COX-2 and membrane-bound PGE synthetase are upregulated in macrophages in atheromatous plaques of humans and induce expression of matrix metalloproteinase-9 and proposed that this pathway leads to plaque instability (37, 38). It is interesting in this context that PGI₂ can suppress expression of...
This matrix metalloproteinase isoform in vitro and in vivo (39, 40). Lesion rupture, when it occurs in vivo, then precipitates thrombosis, which is further accelerated in the absence of IP. Disruption of IP is known to increase the risk of thrombosis (20). Thus, PGI1 appears to exert important inhibitory actions on the initiation and progression of atherosclerosis, and the reduction in PGI1 in the presence of normal TXA2 formation is likely to lead an increased risk of atherosclerosis and thrombosis. Currently, an important question concerning COX-2 inhibitors is whether the selective reduction in PGI1 increases the risk of atherosclerosis. Our findings support that conclusion. However, our findings cannot be directly extrapolated to the clinical outcome of patients treated with COX-2 inhibitors. Although the majority of PGI1 under basal conditions is derived from COX-2 catalysis, both COX-1 and COX-2 contribute to the increase in PGI1 in patients with atherosclerosis as well as in apoE–/– mice (3, 17), and selective inhibition of COX-2 usually results in only partial inhibition of PGI1 production (15–17).

In addition, TXA2 can be derived also from COX-2 in atherosclerotic plaques. COX-2 is expressed by monocytes/macrophages in mouse atherosclerotic lesions (15). Macrophages contain TX synthase and release large amounts of TXA2 when transformed into foam cells with modified LDL (41).

What, then, are the underlying mechanisms of the actions of TXA2 and PGI1 in atherogenesis? Activation and inhibition of blood platelets by TXA2 and PGI1, respectively, may certainly be one of the mechanisms. Activated platelets were found in the circulating blood of patients with atherosclerosis (42–44) and hypercholesterolemia (45, 46). We have examined this issue by using whole-blood flow cytometry for thrombin-induced P-selectin expression in platelets (32). This method has been used frequently to evaluate platelet reactivity in patients with various cardiovascular disorders (33). Our analysis has revealed that platelets of apoE–/–/TP–/– and apoE–/–IP–/– mice have lower and higher reactivity, respectively, than those of apoE–/– mice, which is consistent with the atherosclerotic phenotypes observed in the three strains of mice.

Here, we have further examined effects of TP or IP disruption on expression of adhesion molecules on ECs. Adhesion molecules on ECs play important roles in the migration of monocytes/macrophages through the EC monolayer and the initiation of atheromatous plaques. Indeed, ICAM-1 is strongly expressed in atherosclerotic plaques of humans (47) and the level of soluble ICAM-1 correlates with the severity of atherosclerosis (48). In apoE–/– mice, ICAM-1 expression is high in atherosclerosis-prone sites of the aorta, and deficiency in ICAM-1 in apoE-deficient mice significantly reduces atherosclerotic lesions (49). We have found that ICAM-1 expression on ECs overlying the plaques of apoE–/–TP–/– mice is significantly lower, while that of apoE–IP–/– mice is significantly higher, than ICAM-1 expression in apoE–/– mice (Figure 3). The changes in the ICAM-1 expression in the presence of TP or IP deficiency are consistent with the reported in vitro actions of TXA2 and PGI1. ICAM-1 expression is induced by proinflammatory cytokines from activated macrophages such as TNF-α or IL-1β (50). Signaling from PGI1 to IP is known to inhibit TNF-α production by activated macrophages (21) and to reduce IL-1-induced ICAM-1 expression on ECs (51). In contrast, stimulation of TP induces ICAM-1 expression in cultured ECs in vitro (52, 53), suggesting that TXA2 formed in situ in atheromatous plaques acts on ECs to induce ICAM-1 expression to amplify atherogenesis. Interestingly, TXA2 and PGI1 appear to have effects opposite to those of ICAM-1 on the expression of PECAM-1 on the plaque ECs, which was up- and downregulated in apoE–/–TP–/– and apoE–/–IP–/– mice, respectively. PECAM-1 was first described as an adhesion molecule essential in the transmigration of leukocytes through endothelial monolayer (54). However, recent analyses of PECAM-1–/– mice in various models showed that PECAM-1 deficiency did not block but instead enhanced leukocyte accumulation at inflammation sites (55–57). Given its intracellular domain, PECAM-1 is now suggested to be an inhibitory signaling molecule (58). Intriguingly, regulation of PECAM-1 expression is opposite to that of ICAM-1. For example, a previous report showed that the expression of PECAM-1 and ICAM-1 on cultured human umbilical vein ECs was down- and upregulated, respectively, after activation with TNF-α plus IFN-γ (59). Such opposite modes of expression may explain the changes in the patterns of ICAM-1 and PECAM-1 expression found in the atherosclerosis phenotypes of apoE–/–TP–/– and apoE–/–IP–/– mice.

The above findings on the reactivity of platelets and the expression of adhesion molecules in ECs in apoE–/–, apoE–/–TP–/–, and apoE–/–IP–/– mice suggest that TP or IP deficiency can affect the interaction of ECs with platelets and leukocytes. We examined this issue by intravital microscopy. Although we did not detect significant platelet adhesion to the blood vessels of any of the three lines of mice under basal conditions, we found significant leukocyte adherence to the wall of the common carotid artery in apoE–/–IP–/– mice. This may be relevant to the higher platelet reactivity and enhanced ICAM-1 expression in this line of animals. Platelet P-selectin is suggested to play an important role in mediating the leukocyte-EC interaction (60). It may be also relevant to the EC disruption observed in apoE–/–IP–/– mice.

In conclusion, using the IP-deficient and TP-deficient mice, we were able to evaluate separately the contributions of PGI1 and TXA2 to the development of atherosclerosis. The information presented here will aid in the interpretation of clinical findings and the evaluation of risk in atherosclerotic patients treated with various drugs modulating the arachidonate cascade. Our findings also indicate that the administration of PGI mimetics and TP antagonists may be useful in the prevention of atherosclerosis. This line of genetic approach may also help to identify the contributions of PGs other than PGI1 and TXA2 to atherosclerosis.

Methods

Generation of apoE–/–TP–/– and apoE–/–IP–/– double-KO mice. ApoE–/– mice (129Olα × C57BL/6 mixed background) were a generous gift from Edward M. Rubin (University of California at Berkeley, Berkeley, California, USA) (4). Mice lacking TP or IP individually were generated as described (19, 20). ApoE–/–, TP–/–, and IP–/– mice were backcrossed 5, 10, and 10 times, respectively, to C57Bl/6CrSlc mice (Japan SLIC). TP–/–IP–/– mice were then crossed-bred with apoE–/– mice. Functional disruption of the gene encoding apoE was confirmed by markedly elevated plasma cholesterol levels. Genotype analyses of apoE–/–, TP–/–, and IP–/– mice were performed by PCR using genomic DNA isolated from tail tip samples as a template. PCR analysis was performed for apoE alleles with the sense primers exon2 (5′-GTTGCTGTGGTGTCAATTGCTGCA-3′) and Neo1 (5′-ATGGGATCGGCATATGAACA-3′) for WT and mutant alleles, respectively, and the antisense primer exon3 (5′-TCAGTTCTTGTGATCAGTGGAGC-3′), for TP alleles with the sense primers ML139 (5′-AAGGTAATGTACGACACCATCCTTC-3′) and Neo2 (5′-TGTATGACTGGAGGACTTTG-3′) for TP alleles with the sense primers ML138 (5′-AAGCCTGGTGGTCCAGGACTTTG-3′) and for IP alleles with the sense primer CY37 (5′-GTATCTTTTCAGCTTTGAGGACTTTG-3′) and.
the antisense primers CY41 (5′-GAGCAGAAAAATTCGCCAGGGCTT-3′) and Neo17 (5′-TGAGCCTCTCTGCTTTTAT-3′) for WT and mutant alleles, respectively (Figure 1A). Reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 10% DMSO, 0.25 mM dNTPs, 20 pmol of each primer, and 1 U of Taq DNA polymerase (Toyobo) in a total volume of 20 μL. After a denaturation step at 94°C for 3 minutes, 35 cycles of the amplification step (94°C for 60 seconds, 58°C for 60 seconds, and 72°C for 80 seconds) were carried out, followed by a final elongation step of 3 minutes at 72°C. For apoE alleles, primers exon2 and exon3 amplify a 0.7-kb WT allele fragment, and primers Neo1 and exon3 amplify a 0.4-kb mutant allele fragment. For TP alleles, primers ML139 and ML136 amplify a 0.9-kb WT allele fragment, and primers Neo2 and ML136 amplify a 1.1-kb mutant allele fragment (Figure 1A). For IP alleles, primers CY37 and CY41 amplify a 1.3-kb WT allele fragment, and primers CY37 and Neo17 amplify a 0.9-kb mutant allele fragment (Figure 1A). Mouse was kept on a 12-hour light/dark cycle and were fed a normal chow diet (F2, Funabashi Farm). Food and water were available ad libitum. All experiments were performed in male mice. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

Preparation of mouse platelets and platelet aggregation assay. Platelet aggregation was examined as described previously (61). Blood (1.0 ml) was drawn by cardiac puncture of ether-anesthetized mice with a syringe containing 50 μL of 3.8% trisodium citrate. Blood pooled from 3–4 animals was diluted with an equal volume of modified Tyrode-HEPES buffer, pH 7.4 (20 mM HEPES, 140 mM NaCl, 5 mM MgCl2, and 5 mM KCl). Platelet-rich plasma (PRP) was prepared by centrifugation at 160 g for 10 minutes at room temperature. The number of platelets in the PRP was determined with a hemocytometer (NBS Hema Tracer 601; Tokyo Koden). 1-BOP, a TP agonist, was used to activate platelets, and cicaprost, an IP agonist, was used in a control experiment. Platelet-poor plasma was obtained by centrifugation at 1,500 g for 10 minutes at 0°C. Platelet-poor plasma was diluted with an equal volume of modified Tyrode-HEPES buffer containing 50 μM EGTA, as described previously (62, 63). The aorta was opened of more than 1.063 g/ml cholesterol; LDLC is the difference between TC and VLDLC; HDLC is cholesterol with a density greater than 1.019 g/ml. Blood (1.0 ml) was drawn by cardiac puncture of ether-anesthetized mice with a syringe. Platelet-rich plasma (PRP) was prepared by centrifugation at 160 g for 10 minutes at room temperature. The number of platelets in the PRP was determined with a hemocytometer (NBS Hema Tracer 601; Tokyo Koden). 1-BOP, a TP agonist, was used to activate platelets, and cicaprost, an IP agonist, was used to inhibit platelet aggregation.

Lipid and lipoprotein analyses. Blood (1.0 ml) was drawn by cardiac puncture of ether-anesthetized mice into a tube containing EDTA (final concentration, 5 mM). Plasma was isolated by centrifugation at 1,500 g for 10 minutes and was maintained at 4°C. Plasma cholesterol and triglyceride were measured using Toyobo enzymatic assay kits (Toyobo). For quantification of the cholesterol content of each lipoprotein, lipoproteins were separated at buoyant densities of 1.019 g/ml and 1.063 g/ml by ultracentrifugation. VLDLC is the difference between TC and cholesterol with a density greater than 1.019 g/ml; HDLC is cholesterol with a density of more than 1.063 g/ml cholesterol; LDL is the difference between TC and the sum of VLDLC and HDLC.

Quantification of atherosclerosis. Atherosclerotic lesions were quantified by en face analysis of the whole aorta and by cross-sectional analysis of the proximal aorta and the innominate artery. For en face preparations of the aorta, a cannula was inserted into the left ventricle and the aortic tree was fixed by perfusion for 10 min with ice-cold PBS containing 4% paraformaldehyde (PFA), 5% sucrose, 20 μM butylated hydroxytoluene, and 2 μM EDTA, as described previously (62, 63). The aorta was opened longitudinally, from the heart to the iliac arteries, while still attached to the aorta. The sections were then incubated overnight at 4°C with a 1:200 dilution of mouse macrophages (Accurate Chemical and Scientific Co.); a 1:200 dilution of mouse ICAM-1, labeled with Texas Red (BD); and a 1:200 dilution of rat vWF staining, endogenous peroxidase activity was blocked by incubation of sections at 4°C for 30 minutes with 0.3% (volume/volume) H2O2 in PBS. The sections were then incubated overnight at 4°C with a 1:200 dilution of rat mAb against mouse macrophages (Accurate Chemical and Scientific Co.); a 1:200 dilution of mouse 1A4 mAb against human α-smooth muscle actin, labeled with FITC (Dako); a 1:200 dilution of armenian hamster mAb against mouse ICAM-1, labeled with Texas Red (BD); and a 1:200 dilution of rat mAb against mouse PECAM-1, labeled with FITC or Texas Red (BD). Sections incubated with MOMA-2 antibody were then washed and incubated with 1:400 dilution of goat anti-rat IgG, labeled with Texas Red (BD). For vWF staining, endogenous peroxidase activity was blocked by incubation of sections at 4°C for 30 minutes with 0.3% (volume/volume) H2O2 in PBS. The sections were then incubated overnight at 4°C with a 1:200 dilution of mouse mAb against human vWF, labeled with HRP (Sigma-Aldrich). After a thorough washing, staining was developed with diaminobenzidine followed by counterstaining with hematoxylin. Ten sections obtained every 18 μm from aortic arch were used for quantification of the macrophages and SMCs and EC density of the lesions with Image-Pro Plus software. The macrophages and SMCs were quantified by measurement of the area that stained positive for the respective markers, as described previously (7). EC density was determined by the ratio of the vWF-positive luminal surface length to the total luminal surface length of each cross-sectional plaque. The average of the 10 sections was taken to represent 1 animal, and the means of the averages from each group were compared.

For cross-sectional analysis of the aorta, hearts were isolated from mice sacrificed by cervical dislocation, were washed in PBS, and were embedded in OCT compound. The OCT-embedded hearts were sectioned with a cryostat, and 6-μm sections in the proximal aorta were obtained sequentially beginning at the aortic valve. Sections were transferred onto a Superfrost slide (Matsunami) and were stained with oil red O followed by counterstaining with hematoxylin (4). Ten sections obtained every 36 μm from the aortic sinus were used for quantification of lesion areas with Image Pro Plus software (Media Cybernetics). The average lesion area of the ten sections from each heart was taken as a value to represent that animal, and the means of the average lesion areas from each group were compared as described previously (64, 65).

Atherosclerotic lesions in the innominate artery were quantified by cross-sectional analysis. Innominate arteries were isolated from 45-week-old male mice sacrificed by cervical dislocation, were washed in PBS, and were embedded in OCT compound. OCT-embedded innominate arteries were sectioned with a cryostat and 8-μm sections were obtained sequentially. Sections were transferred onto a Superfrost slide and were stained with hematoxylin and eosin. Ten sections obtained every 80 μm were used for quantification of lesion areas with Image Pro Plus software. The average lesion area of the 10 sections from each innominate artery was taken as a value to represent that animal and the means of the average lesion areas from 10 mice were compared.

Immunohistochrometry. For cross-sectional analyses, the aortic tree was perfused with ice-cold PBS containing 5 mM EDTA via a cannula inserted into the left ventricle for 10 minutes. The aortic arch was isolated, embedded in OCT compound, and sectioned at a thickness of 6 μm with a cryostat. Sections containing atherosclerotic plaques were identified by microscopy. These sections were then fixed in 4% PFA at 4°C for 10 minutes, were immersed in PBS for 5 minutes for rehydration of the tissues, and were blocked overnight at 4°C with 2% skim milk (BD) in PBS. For evaluation of the abundance of macrophages and SMCs and the expression of ICAM-1 (CD54) and PECAM-1 (CD31) in the lesions, sections were incubated overnight at 4°C with a 1:200 dilution of rat MOMA-2 mAb against mouse macrophages (Accurate Chemical and Scientific Co.); a 1:200 dilution of mouse 1A4 mAb against human α-smooth muscle actin, labeled with FITC (Dako); a 1:200 dilution of armenian hamster mAb against mouse ICAM-1, labeled with Texas Red (BD); and a 1:200 dilution of rat mAb against mouse PECAM-1, labeled with FITC or Texas Red (BD). Sections incubated with MOMA-2 antibody were then washed and incubated with 1:400 dilution of goat anti-rat IgG, labeled with Texas Red (BD). For vWF staining, endogenous peroxidase activity was blocked by incubation of sections at 4°C for 30 minutes with 0.3% (volume/volume) H2O2 in PBS. The sections were then incubated overnight at 4°C with a 1:200 dilution of mouse mAb against human vWF, labeled with HRP (Sigma-Aldrich). After a thorough washing, staining was developed with diaminobenzidine followed by counterstaining with hematoxylin. Ten sections obtained every 18 μm from aortic arch were used for quantification of the macrophages and SMCs and EC density of the lesions with Image-Pro Plus software. The macrophages and SMCs were quantified by measurement of the area that stained positive for the respective markers, as described previously (7). EC density was determined by the ratio of the vWF-positive luminal surface length to the total luminal surface length of each cross-sectional plaque. The average of the 10 sections was taken to represent 1 animal, and the means of the averages from each group were compared.
For the en face analysis, the aortic tree was first washed by perfusion with ice-cold PBS containing 5 mM EDTA and then was fixed by perfusion with ice-cold PBS containing 4% PFA via a cannula inserted into the left ventricle, each perfusion for 10 minutes. The aortic arch was isolated and opened longitudinally. En face preparations were blocked overnight at 4°C with 2% skim milk in PBS and were incubated overnight at 4°C with a 1:500 dilution of rat mAb against mouse PECAM-1, labeled with FITC, and armenian hamster mAb against mouse ICAM-1, labeled with Texas Red. Because activation of ECs occurs on the “shoulder” of plaques (66), five images (1,024 × 1,024 pixels/image) were obtained randomly from the EC monolayer on the “shoulder” of plaques with a Bio-Rad MRC-1024 confocal microscope. The average pixel intensity of the five images was taken as a value to represent that animal, and the means of the average pixel intensity from each group were compared as described previously (60).

Silver nitrate staining of en face endothelial cells. The aortic tree was washed, stained, and fixed as described previously (67, 68) by successive perfusion in the following solutions: 10 ml of 5% glucose; 4 ml of 0.25% silver nitrate; 2 ml of 5% glucose; 8 ml of 3% cobalt bromide and 1% ammonium bromide; 2 ml of 5% glucose; 4 ml of 4% PFA; 10 ml of distilled water; 2 ml of hematoxylin; and 10 ml of distilled water. The aortic arch was isolated, opened longitudinally, and mounted with the endothelium upward on a Superfrost slide.

Flow cytometry for platelet reactivity. Platelet reactivity was examined by whole-blood flow cytometry (32, 33). Blood (1.0 ml) was drawn by cardiac puncture of ether-anesthetized mice with a syringe containing 50 μl of 3.8% trisodium citrate. Within 10 minutes of being drawn, the blood was diluted 1:4 in modified Tyrode-HEPES buffer, pH 7.4, and the diluted blood was activated at 37°C for 10 minutes with 0.1–0.5 U/ml thrombin, incubated at room temperature for 30 minutes with a 1:100 dilution of rat mAb against mouse P-selectin, labeled with FITC, and fixed at 4°C for 2 hours with ice-cold PBS containing 1% PFA. Samples were then analyzed using a FACSVerse flow cytometer (BD).

Intravital microscopy. Leukocyte-EC interaction was examined by intravital microscopy using rhodamine 6G that stained in vivo leukocytes, as described previously (29, 60, 69). Five male mice 25–30 weeks of age were used for each strain. Rhodamine 6G was injected i.v., and the numbers of leukocytes rolling on and adhering to the wall of the common carotid artery were examined “off-line” during video playback analysis. A leukocyte was defined as rolling if it migrated along the vessel wall at a rate less than 200 μm/s and as adhering if it remained stationary for more than 20 seconds. We counted the number of leukocyte rolling and adhering in the artery per microscope field (×100) and expressed the results as the number of leukocytes observed per mm² area per minute.

Statistical analysis. Data are presented as means ± SEM. Comparison of two groups was analyzed by Student’s t test. For comparison of more than two groups with comparable variances, one-way ANOVA was performed, followed by Tukey’s t test to evaluate pair-wise group differences. An associated probability (P value) of less than 0.05 was considered significant. Analyses were performed with the use of GraphPad Software Prism 3.0.

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