hypercholesterolemia should be determined individually according to their physical activities. It is noted that the elderly are more susceptible to drug-related adverse effects than the younger since renal and liver functions, required for metabolizing drugs, in the elderly are relatively weaker.

Keywords: cardiovascular event, elderly, hypercholesterolemia, Japanese, statin.

Introduction

It is well known that cardiovascular events occur in elderly people more frequently than in the younger population. It is also known that the incidence of these events increases as serum cholesterol levels are elevated. In Japan, populations of elderly people are rapidly increasing and serum cholesterol levels have been clearly rising in all ranges of ages probably due to westernization of our dietary habits.[1] Therefore, a rapid increase in atherosclerotic diseases is anticipated in Japan, especially in the elderly, without appropriate prevention.

Data obtained in many clinical studies performed in Western countries have demonstrated that cholesterol-lowering therapy with HMG-CoA reductase inhibitors, statins, reduces cardiovascular events by 26–37%.[2-4] Therefore, therapeutic intervention to control serum cholesterol levels is widely accepted. So far, guidelines for controlling cholesterol levels have been established in several countries, such as ATPIII (http://www.nhlbi.nih.gov/guidelines/cholesterol/atp_iii.htm) in the USA. Since the incidence of cardiovascular events in the Japanese population is clearly lower than that in Western countries, establishment of the Japanese guideline has been considered necessary. The first Japanese guideline was established by the Japanese Atherosclerosis Society in 1997 and it has been revised in 2002 (http://jas.umin.ac.jp). Since the subjects for the guideline are those aged ≤ 65 years, the guideline for elderly Japanese has been expected to be established.

In 1996–99, the research group for 'Establishing Japanese guidelines for treating atherosclerotic diseases in the elderly' was organized as part of the Comprehensive Research on Aging and Health conducted by the Japanese Ministry for Health, Labour and Welfare and the first guideline was proposed in 1999 (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). In this guideline, the target cholesterol levels for the elderly were recommended to be 20 mg/dL higher than those for the younger population, based on the comparison of relative risk increase in relation to serum cholesterol levels between younger people and the elderly (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). Since then, several important clinical datasets in Western countries and results of studies conducted in Japan,[2-4] such as the KLIS,[5,6] the J-LIT and PATE have been produced.[7-9] Therefore, the research group was again organized in 1999–2002 in order to conduct a research project entitled 'Long-term prognosis of the elderly with hyperlipidemia' (chaired by T. Kita) as a part of the Comprehensive Research on Aging and Health with a view to re-evaluating the proposed guideline (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). The research group has concluded that serum cholesterol levels in Japanese aged 65–74 years are recommended to be controlled in the same way as for patients aged ≤ 65 years by following the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases (2002) by the Japan Atherosclerosis Society (http://jas.umin.ac.jp/), and that for those aged ≥ 75 years the control levels should be determined individually based on their physical activities (Kita & Matsuzawa *et al.* unpublished report to the Japanese Ministry of Health and Welfare 2002).

Clinical data in Western countries

Secondary prevention studies such as 4S and CARE have been analyzed with a focus on the elderly.[10,11] In both studies, treatment with simvastatin and pravastatin in the elderly patients was as safe and effective for reducing serum cholesterol levels as it was in younger patients.[10,11] In the 4S study, 4444 patients with established coronary heart diseases were divided into simvastatin and placebo groups, and followed for 5.4 years.[10] In this study, simvastatin treatment reduced total cholesterol levels by 26% in the elderly aged 65–70 years and by 25% in younger patients,[10] indicating that the cholesterol lowering effect of simvastatin in the elderly is similar to that in the younger. The relative risk reduction of major coronary events, including coronary artery death and non-fatal myocardial infarction, by simvastatin in the elderly patients was 34%, similar to that in younger patients aged < 65 years.[10] In the CARE study, 4159 patients were divided into pravastatin and placebo groups and followed for 5 years.[11] In this study, pravastatin treatment reduced total cholesterol levels by 19% in the elderly aged 65–75 years and by 20% in patients aged < 65 years,[11] indicating that the cholesterol lowering effect of simvastatin in the elderly is similar to that in younger patients. The relative risk reduction in the elderly group was 39% while that in the younger was 13%.[11] Because of the higher absolute risk and greater effect on risk reduction in the elderly group, the number

needed to treat (NNT) in the 5-year follow-up period in the elderly group was 15 while that in the younger group was 67.[11]

Recently, the results of the PROSPER study have been published.[12] In this study, approximately 5800 high-risk patients aged 70–82 (mean 75 years) with normal total cholesterol levels (mean 217 mg/dL) were divided into pravastatin and placebo groups, and followed for 3 years. In this elderly population, the statin reduced coronary events by 19%. Since the preventive effects by statins become obvious in 1–2 years after starting the medication in many studies,[2-4] the risk reduction ratio in the PROSPER study could be relatively smaller during the 3-year follow-up period.[12] In the ASCOT study, approximately 19 000 hypertensive high-risk patients with total cholesterol levels of ≤ 250 mg/dL (213 mg/dL average), aged 40–79 years (mean 63 years), were assigned into placebo and 10 mg/day atorvastatin groups, and followed for 3 years.[13] The results showed that treatment with atorvastatin reduced coronary events by 36%. The risk reduction in the subgroup aged ≥ 60 years was also 36%, which was similar to that in younger patients aged < 60 years. Thus, it has been demonstrated in studies conducted in Western countries that cholesterol-lowering therapy in the elderly brings similar, or even better, effects in the prevention of coronary events, compared with its effects on younger patients.

It has been demonstrated that cholesterol lowering therapy by statins slowed the narrowing of coronary arteries and reduced intima-media thickness in carotid arteries.[14,15] Thus, cholesterol lowering by statins could stabilize atheromatous plaque, thereby inhibiting the event occurrence.

Clinical data in Japan

The Hisayama study was an epidemiological study conducted in the Hisayama community in Japan.[16] In this study, where 2673 people aged ≥ 40 years were followed from 1988 to 1996, the absolute risk for ischemic heart diseases (myocardial infarction and sudden death) was reported to be 2.3/1000/year and that for cerebral infarction to be 3.1/1000/year.[17]

The J-LIT study was a cohort observational study in Japan. In this study, approximately 50 000 hypercholesterolemic patients aged ≤ 70 years undergoing 5–10 mg/day simvastatin treatment were followed for 6 years. A subanalysis focusing on elderly patients without prior coronary events was performed.[18] In both the elderly group, aged 65–70 years (mean 67 years) and consisting of 9860 patients, and the younger group, aged ≤ 64 years (mean 55 years) and consisting of 32 500 patients, total cholesterol levels were approximately 270 mg/dL at enrollment and 210–220 mg/dL during follow-up periods under simvastatin treatment. Changes in low-density lipoprotein (LDL)-cholesterol levels were also similar: levels of approximately 180 mg/dL at the baseline were reduced to approximately 130 mg/dL in the follow-up periods in both groups. No severe drug-related adverse effects occurred in either group. Thus, statin treatment in the elderly is as safe and effective for reducing serum cholesterol levels as it is in younger patients. The doses of the statin were lower than those used in Western countries, where 20–40 mg/day simvastatin was used.[2]

In the J-LIT study, the incidence of coronary events (sudden cardiac death and acute myocardial infarction) in the elderly was 1.30/1000/year and that in the younger 0.8/1000/year. When occurrence of angina was included in coronary events, the incidence in the elderly was 2.25/1000/year and that in the younger 1.35/1000/year. Cox-biohazard analysis revealed that the relative risks of coronary events increased by 1.7% as serum LDL-cholesterol levels increased by 1 mg/dL, which were similar in both groups.[18] Importantly, in any LDL-cholesterol levels, the absolute risk in the elderly was higher than that in the younger. Generally, coronary events occur twice as often in men as in women, which was also observed in the J-LIT study.[7,8] In the J-LIT study, 35% of the study subjects were male in the younger group and 21% were male in the elderly group.[18] Therefore, upon interpretation of this J-LIT data, the male : female ratio should be considered. Indeed, in male patients, the coronary events (sudden cardiac death and acute myocardial infarction) occurred at a rate of 2.45/1000 patients/year in the elderly and 1.41/1000 patients/year in younger patients.

The KLIS study was planned as a primary prevention study for male patients aged 45–74 years with serum cholesterol levels ≥ 220 mg/dL.[5,6] Enrolled patients were assigned into a conventional therapy group and a pravastatin group, and followed for 5 years. However, since the results of several studies revealed superior effects of statin therapy for the event prevention during the study period, the assignment could not be kept completely. As a result, 2219 cases in the pravastatin group and 1634 cases in the conventional therapy group were analyzed. Coronary events (sudden death, myocardial infarction, coronary intervention and bypass surgery) occurred in 5.95/1000 per year in the conventional therapy group and 5.77/1000 per year in the pravastatin group. Cerebral infarction occurred in 5.15/1000 per year in the conventional therapy group and 4.19/1000 per year in the pravastatin group. In the pravastatin group, 1105 cases were of good compliance for the drug-intake. The relative risk of coronary events plus cerebral infarction of the good-compliance group was 0.57 (0.54–0.98) compared with that of the conventional therapy group. A subanalysis examining those aged ≥ 65 years in this study revealed a tendency similar to that observed in the J-LIT study.[18] Namely, coronary events increased as

serum LDL-cholesterol levels increased in both elderly and younger groups, and the absolute risks in the elderly were higher than those in the younger in any given LDL-levels (Sasaki *et al.* in preparation).

In the PATE study, 665 patients (male ratio 21%) aged ≥ 60 years (mean 73 years) with serum total cholesterol levels of 220–280 mg/dL were followed for 3–5 years (mean 3.9 years) under treatment with low-dose (5 mg) or high-dose (10–20 mg) pravastatin.[9] In this study, events were defined as cerebral bleeding, cerebral infarction, transient ischemic attack, subarachnoid hemorrhage, myocardial infarction, angina pectoris, cardiac failure, arrhythmia, arteriosclerosis obliterance, dissecting aortic aneurysm, and peripheral artery thrombosis. During the follow-up period, acute myocardial infarction occurred in 11 cases (4.2/1000/year). In the patient group without diabetes and with serum cholesterol levels of < 253 mg/dL and triglyceride levels of ≥ 133 mg/dL, the event-free ratio in the high-dose group was significantly higher than that in the low-dose group.

Thus, we could expect similar, or even more beneficial, effects of cholesterol-lowering therapy to reduce cardiovascular events in elderly Japanese compared with those in the younger population, although the studies described above appear to be somewhat indirect. Urgently and absolutely required are complete epidemiological and/or interventional large-scale studies, from which we can definitely estimate the absolute risks and the risk reduction rates in the current Japanese population.

Cerebral infarction and hypercholesterolemia

Cerebral infarction is also a disease that occurs more frequently in the elderly. Cerebral infarction is classified into following three: (i) lacunar infarction caused by small artery occlusion which is correlated with hypertension; (ii) cardiogenic cerebral embolism, which is usually associated with atrial fibrillation; and (iii) cerebral infarction caused by atherothrombotic arterial occlusion. Hypercholesterolemia is considered to be linked to atherothrombotic occlusion.

In the 4S secondary prevention study, simvastatin reduced total strokes by 35%.[2] The data obtained in secondary prevention studies with pravastatin, including the LIPID and CARE studies, have been combined and analyzed.[19] The results demonstrated that pravastatin treatment reduced total strokes by 22% and non-hemorrhagic strokes by 23%.[19] In the ASCOT study, atorvastatin reduced fatal and non-fatal strokes by 27%.[13] In the MRC/BHF Heart Protection Study, where approximately 20 000 high-risk patients aged 40–80 years had been randomly assigned into placebo and simvastatin-treated groups and followed for 5 years, simvastatin reduced ischemic strokes by 29%.[20] In the KLIS study conducted in Japan, the incidence of cerebral infarction was 5.15/1000 per year in the conventional therapy group and 4.19/1000 per year in the pravastatin group.[5,6] In the KLIS study, the incidence of cerebral infarction increased as LDL-cholesterol levels increased in elderly aged ≥ 65 years (Sasaki *et al.* in preparation). In the J-LIT study, the incidence of ischemic cerebrovascular events was 1.41/1000 per year in the subgroup without prior coronary or cerebral infarction (Nakaya *et al.* unpublished data). In both studies, the incidence of ischemic cerebral events was clearly higher in the elderly than that in the younger. Thus, evidence is accumulating to support the preventive effects of serum cholesterol-lowering on the occurrence of cerebral infarction. We may expect risk reduction for not only coronary events but also cerebral infarction in cholesterol-lowering therapy. Although the incidence of coronary events in Japan is much lower compared with that in Western countries, the incidence of cerebrovascular events are similar. Since incidence of cerebrovascular events in Japan is similar to that of coronary events, impact of the prevention of cerebrovascular events is as large as that of coronary events in Japan.

Conclusions: Strategy for treating elderly Japanese with hypercholesterolemia

As reviewed above, the control of serum cholesterol levels appears effective in risk reduction of cardiovascular events in elderly Japanese as well as in the younger population. The incidence of such events in the elderly is generally higher than that in younger people. Therefore, the elderly would be even more suitable subjects for preventative intervention. Although it may take long periods to develop atherosclerosis, the preventive effects for cardiovascular events become apparent in 1–2 years after cholesterol-lowering therapy has started, as demonstrated in many studies.[2,3,11,12,20] Therefore, it is not too late for us to start cholesterol-lowering therapy in the elderly. We have concluded after discussion in the research group 'Long-term prognosis of elderly Japanese with hypercholesterolemia' that we could expand the subjects of the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases by the Japan Atherosclerosis Society (2002) to include elderly Japanese aged ≤ 74 years (Kita & Matsuzawa *et al.* unpublished report to the Japanese Ministry of Health and Welfare 2002). In the guideline, patients are divided into several categories based on risk factors and the target cholesterol levels for each category is indicated (http://jas.umin.ac.jp/). Aging, ≥ 45 years for men and ≥ 55 years for women, is defined as a risk factor. Therefore, the target total cholesterol level for the elderly aged 65–74 years without additional risk factors is to be less

than 220 mg/dL and the target LDL-cholesterol level, less than 140 mg/dL. The target levels become lower when elderly patients possess additional risk factors. As described in the guideline (http://jas.umin.ac.jp/), the control of cholesterol levels should be started by changing life styles, followed by drug therapy when appropriate cholesterol levels are not obtained.

For the elderly aged ≥ 75 years, few data for Japanese are available at the moment. Furthermore, it was reported that all causes of mortality increased in the group with lower total cholesterol levels due to an increase in death from infections and malignant tumors in an investigation in Holland, where people aged ≥ 85 years were enrolled.[21] Furthermore, in the Honolulu Heart Program, Japanese–Americans aged 75–93 years (mean 78 years) with a mean total cholesterol level of 149 mg/dL have been reported to have higher mortality than the other groups with the levels at 178, 199 and 232 mg/dL.[22] The physical and nutritional conditions of the highly-aged elderly are various and low cholesterol levels may reflect their worsened health conditions. Therefore, we concluded that, for the highly-aged elderly ≥ 75 years, the target cholesterol levels should be determined individually according to physical and nutritional factors, although a higher absolute risk of cardiovascular events would be expected in the elderly aged ≥ 75 years.

Finally, we again emphasize that physicians should be more careful in their use of drugs in elderly patients since physiological functions of the elderly, such as renal and liver functions required for metabolizing drugs, are not as good as those of the younger patients.

The recommended strategy for treatment for elderly Japanese with hypercholesterolemia

Patients aged 65–74 years

Follow the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases by the Japan Atherosclerosis Society (2002) (http://jas.umin.ac.jp/).

Patients aged ≥ 75 years

The target values of total and LDL-cholesterol levels should be determined individually.

Points of consideration for treatment of elderly with hypercholesterolemia

1 Cholesterol-lowering therapy reduces relative risk of coronary events in not only the younger but also in the elderly to a similar extent.
2 The elderly would be even more suitable subjects of lipid-lowering therapy, since the absolute risk in the elderly is higher than that in the younger.
3 The elderly might be more susceptive to drug-related adverse effects than the younger since renal and liver functions, required for metabolizing drugs, in the elderly are weaker.

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Research article

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*$^{-/-}$*IP*$^{-/-}$ mice showed partial endothelial disruption and exhibited enhanced expression of ICAM-1 and decreased expression of platelet endothelial cell adhesion molecule 1 (PECAM-1) in the overlying endothelial cells compared with those of *apoE*$^{-/-}$*TP*$^{-/-}$ 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 specific COX inhibitors. Among prostanoids, PG I_2/prostacyclin (PGI_2) 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 vasodilator, 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 prostanoid 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 constitu-

Nonstandard abbreviations used: EC, endothelial cell; HDLC, HDL-cholesterol; IP, PGI receptor; LDLC, LDL-cholesterol; LDLR, LDL receptor; PECAM-1, platelet endothelial cell adhesion molecule 1; PFA, paraformaldehyde; PGI_2, PG I_2/prostacyclin; PRP, platelet-rich plasma; SMC, smooth muscle cell; TC, total cholesterol; TG, total triglyceride; TP, TXA receptor; TX, thromboxane; VLDLC, VLDL-cholesterol; vWF, von Willebrand factor.

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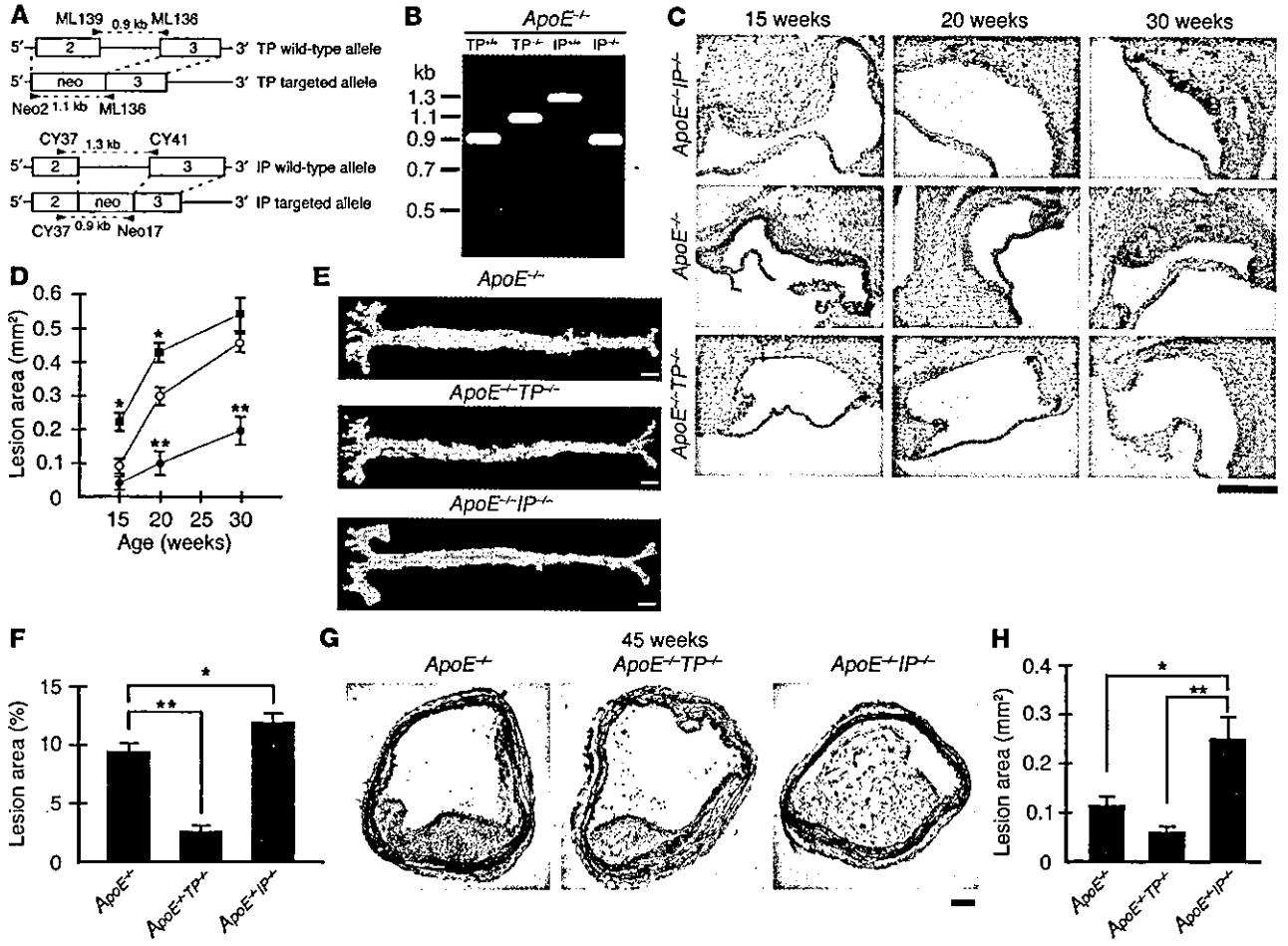

Figure 1

Generation and atherosclerotic lesions of *apoE*$^{-/-}$*TP*$^{-/-}$ and *apoE*$^{-/-}$*IP*$^{-/-}$ mice. (**A**) Strategy for PCR analysis of WT and targeted alleles of TP and IP. Primers are shown by arrowheads. Amplified fragments are shown by broken lines. Neo, neomycin-resistance gene. (**B**) Representative PCR for TP and IP alleles of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice. (**C**) Representative oil red O staining of aortic sinus sections of *apoE*$^{-/-}$ (middle), *apoE*$^{-/-}$*TP*$^{-/-}$ (lower), and *apoE*$^{-/-}$*IP*$^{-/-}$ (upper) mice. Scale bar: 200 μm. (**D**) Time course of atherosclerotic lesion development in *apoE*$^{-/-}$ (open circles), *apoE*$^{-/-}$*TP*$^{-/-}$ (filled circles), and *apoE*$^{-/-}$*IP*$^{-/-}$ (filled squares) mice. Data are means ± SEM (n = 10 for 15-week-old *apoE*$^{-/-}$ and *apoE*$^{-/-}$*IP*$^{-/-}$ and 20-week-old *apoE*$^{-/-}$ and *apoE*$^{-/-}$*TP*$^{-/-}$ male mice; n = 6 for 15-week-old *apoE*$^{-/-}$*TP*$^{-/-}$, 20-week-old *apoE*$^{-/-}$*IP*$^{-/-}$, and 30-week-old *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice). *P < 0.05 and **P < 0.01 versus *apoE*$^{-/-}$ mice. (**E**) Representative Sudan IV staining of en face preparations of aortas from *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice at 20 weeks of age. Scale bars: 2 mm. (**F**) Quantification of en face atherosclerotic lesions in *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$ and *apoE*$^{-/-}$*IP*$^{-/-}$ mice at 20 weeks of age. Data are means ± SEM (n = 5 each). *P < 0.05 and **P < 0.01 for bracketed comparisons. (**G**) Representative hematoxylin and eosin staining of innominate artery sections of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice at 45 weeks of age. Scale bar: 20 μm. (**H**) Quantitative analysis of innominate atherosclerotic areas in *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice at 45 weeks of age. Data are means ± SEM (n = 10 each). *P < 0.05 and **P < 0.01 for bracketed comparisons.

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,

Table 1
Plasma cholesterol and triglyceride levels

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

All data are shown as mean ± SEM. [A]$P < 0.01$ versus C57BL/6. [B]$P < 0.05$ versus C57BL/6.

supporting a view that both COX-1 and COX-2 contribute to PGI_2 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 prostanoids, including PGI_2 and TXA_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 prostanoid by the use of COX inhibitors, because each isoform is capable of producing more than one type of prostanoid 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 atherogenesis found in $LDLR^{-/-}$ mice reconstituted with $COX\text{-}2^{-/-}$ fetal liver cells (15).

In order to conquer these limitations, we have examined the development of atherosclerosis in mice deficient in prostanoid receptors for individual molecules (TXA_2 and PGI_2). TXA_2 and PGI_2 xert 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 (Figure 1, A and B) and was confirmed by examination of the platelet response to a TP or IP agonist (data not shown). The TP agonist I-BOP induced aggregation of platelets from $apoE^{-/-}$ mice but not of platelets from $apoE^{-/-}TP^{-/-}$ mice, whereas thrombin-induced aggregation occurred similarly in platelets from $apoE^{-/-}$ and $apoE^{-/-}TP^{-/-}$ mice. In contrast, the IP agonist cicaprost inhibited I-BOP-induced platelet aggregation in $apoE^{-/-}$ mice and this response was lost in $apoE^{-/-}IP^{-/-}$ platelets. The apoE deficiency in these mice was verified by measurement 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 $apoE^{-/-}$, $apoE^{-/-}TP^{-/-}$, 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 μm 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 (0.089 ± 0.015 mm^2 and 0.290 ± 0.015 mm^2; $P < 0.05$, 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 (0.290 ± 0.015 mm^2 and 0.438 ± 0.025 mm^2; $P < 0.01$, 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

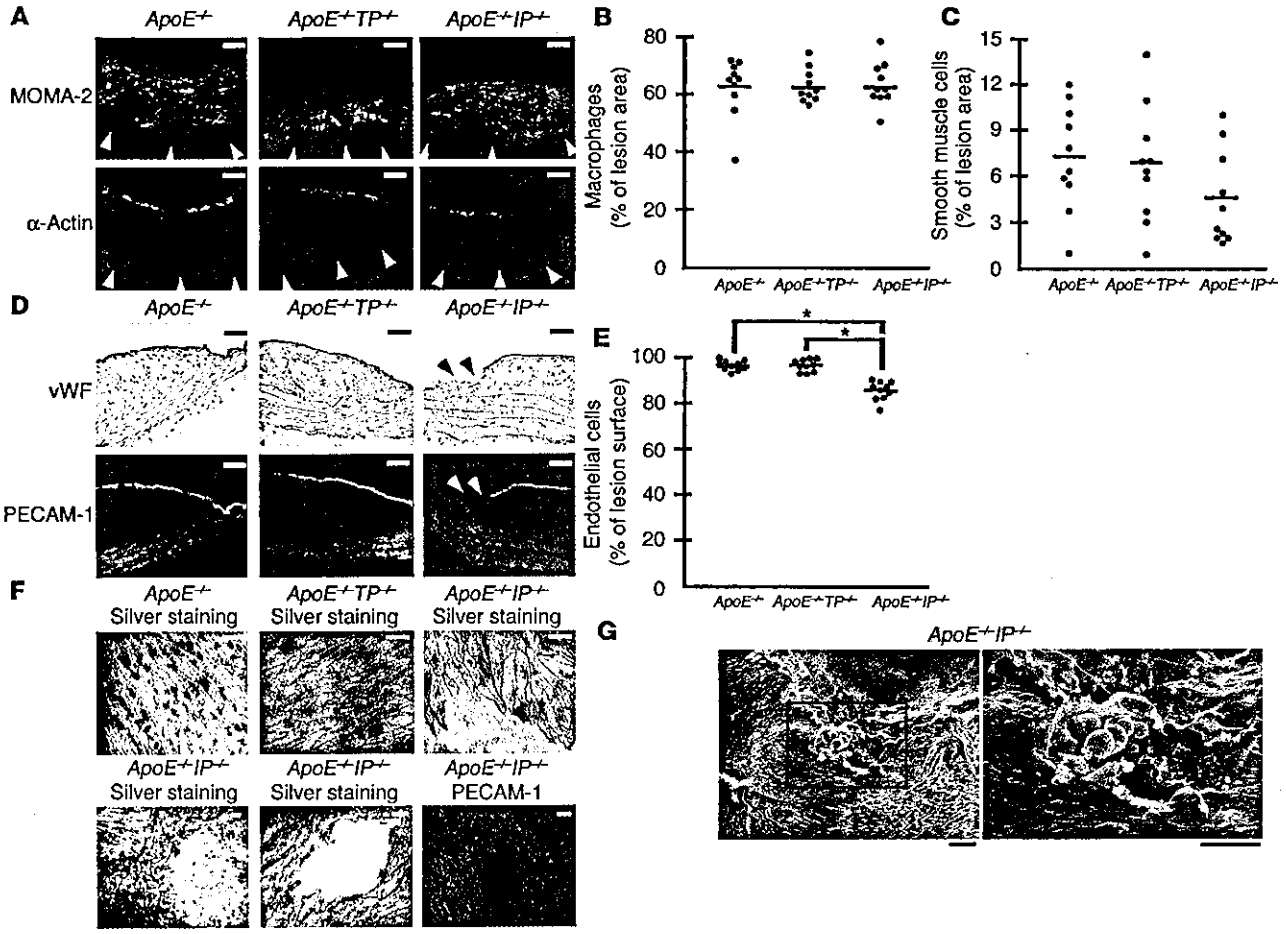

Figure 2

Effects of TP or IP deficiency on the abundance of macrophages and SMCs and EC integrity in aortic arch lesions of apoE-deficient mice at 20 weeks of age. (**A**) Representative immunostaining of macrophages and SMCs in aortic arch lesions of *apoE*$^{-/-}$ (left panels), *apoE*$^{-/-}$*TP*$^{-/-}$ (middle panels), and *apoE*$^{-/-}$*IP*$^{-/-}$ (right panels) mice. Lesions were stained with specific antibodies for macrophages (MOMA-2; upper) and for SMCs (α-actin; lower). White arrowheads indicate the external elastic lamina. Scale bars: 20 μm. (**B** and **C**) Quantitative analysis of the abundance of macrophages (**B**) and SMCs (**C**) in aortic arch lesions of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice. Data are means ± SEM (n = 10 each). (**D**) Representative immunostaining of ECs in aortic arch lesions of *apoE*$^{-/-}$ (left panels), *apoE*$^{-/-}$*TP*$^{-/-}$ (middle panels), and *apoE*$^{-/-}$*IP*$^{-/-}$ (right panels) mice. Cross-sections were stained with specific antibodies for ECs (vWF, upper, and PECAM-1, red, lower) and SMCs (α-actin, green, lower). Black and white arrowheads indicate the site of endothelial disruption. Scale bars: 20 μm. (**E**) Quantitative analysis for endothelial integrity in aortic arch lesions of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice by measurement of the vWF-positive signals overlying aortic lesions. Data are means ± SEM (n = 10 each). *P < 0.05 for bracketed comparisons. (**F**) Representative en face staining of aortic arch lesions of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice. En face staining of aortic arch lesions with silver nitrate or anti–PECAM-1 was performed. Scale bars: 10 μm. (**G**) Representative scanning electron micrographs of aortic arches of *apoE*$^{-/-}$*IP*$^{-/-}$ mice. The right panel shows a higher magnification of the boxed area in the left panel. Scale bars: 50 μm.

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

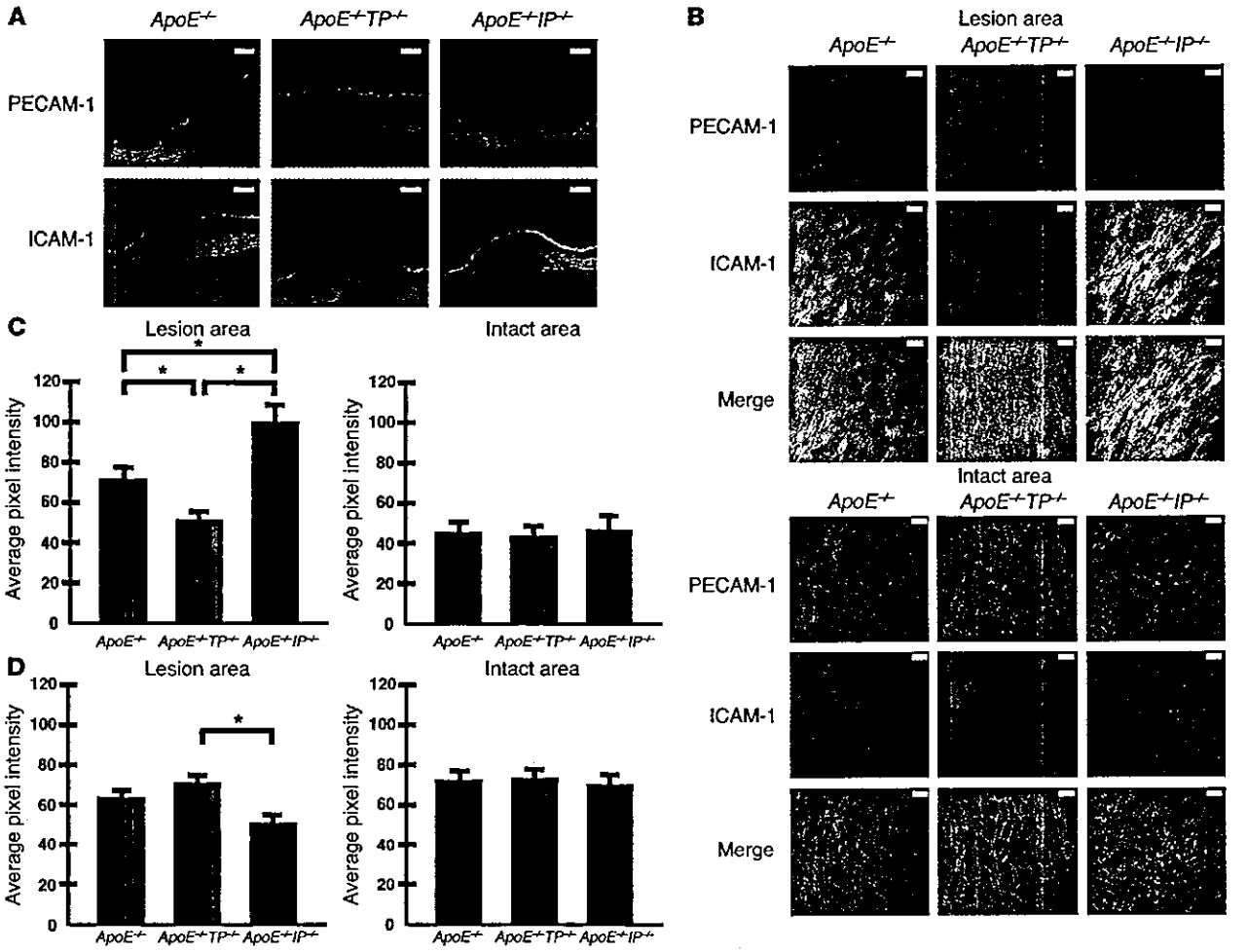

Figure 3
Effects of TP or IP deficiency on ICAM-1 and PECAM-1 expression in the ECs overlying atheromatous lesions of apoE-deficient mice. (**A**) Representative immunostaining for ICAM-1 and PECAM-1 in cross-sections from *apoE*$^{-/-}$ (left panels), *apoE*$^{-/-}$*TP*$^{-/-}$ (middle panels), and *apoE*$^{-/-}$*IP*$^{-/-}$ (right panels) mice. Mice were sacrificed at 20 weeks of age. Cross-sections were stained with specific antibodies against ICAM-1 (red; lower panels), PECAM-1 (red; upper panels), and smooth muscle α-actin (green). Scale bars: 20 μm. (**B**) Representative immunostaining of aortic arch lesions and neighboring intact areas for ICAM-1 and PECAM-1 in en face preparations of *apoE*$^{-/-}$ (left panels), *apoE*$^{-/-}$*TP*$^{-/-}$ (middle panels) and *apoE*$^{-/-}$*IP*$^{-/-}$ (right panels) mice. En face preparations were stained with specific antibodies against ICAM-1 (middle panels) and PECAM-1 (upper panels). Mice were sacrificed at 20 weeks of age. Scale bars: 10 μm. Merge, merged images. (**C** and **D**) Quantification of ICAM-1 (**C**) and PECAM-1 (**D**) expression in ECs overlying aortic arch lesions and neighboring intact areas of *apoE*$^{-/-}$, *apoE*$^{-/-}$*TP*$^{-/-}$, and *apoE*$^{-/-}$*IP*$^{-/-}$ mice. Data are means ± SEM (n = 8 each). $^{*}P < 0.05$ and $^{**}P < 0.01$ for bracketed comparisons.

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*$^{-/-}$ 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 represented 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

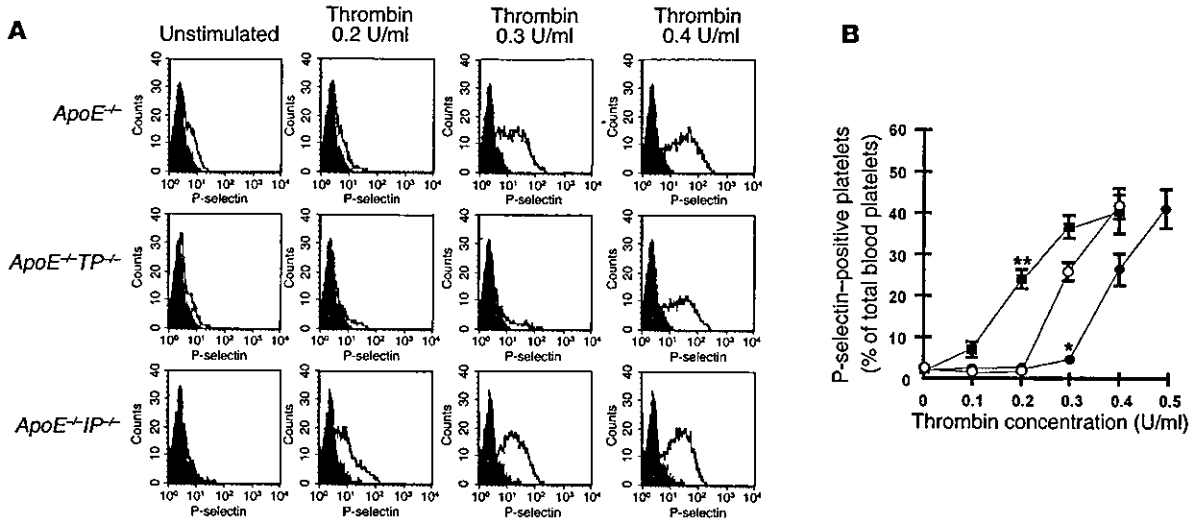

Figure 4

Platelet reactivity for thrombin-induced surface expression of P-selectin. (**A**) Representative histograms of thrombin-induced P-selectin expression in platelets from *apoE*$^{-/-}$ (upper panels), *apoE*$^{-/-}$*TP*$^{-/-}$ (middle panels), and *apoE*$^{-/-}$*IP*$^{-/-}$ (lower panels) mice. Platelets were either left unstimulated or were stimulated with 0.2, 0.3, or 0.4 U/ml of thrombin. They were then labeled with FITC-conjugated anti–P-selectin and were analyzed by flow cytometry. Filled histograms indicate background signal. (**B**) Quantification analysis. Concentration-dependent effect of thrombin for P-selectin expression was determined in platelets from *apoE*$^{-/-}$ (open circles), *apoE*$^{-/-}$*TP*$^{-/-}$ (filled circles), and *apoE*$^{-/-}$*IP*$^{-/-}$ (filled squares) mice. Data are means ± SEM (n = 9 each). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus *apoE*$^{-/-}$ mice.

of SMC proliferation by a PGI_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 EC layer over the plaques. There was occasional loss of staining of these two markers in the plaques of *apoE*$^{-/-}$*IP*$^{-/-}$ mice, especially on the "shoulder" of atherosclerotic lesions (Figure 2D). In contrast, no such irregularity in EC staining on the plaque surface was seen in *apoE*$^{-/-}$ and *apoE*$^{-/-}$*TP*$^{-/-}$ mice. Quantification of vWF staining on the plaque surface revealed a significant reduction in *apoE*$^{-/-}$*IP*$^{-/-}$ mice (85.5% ± 0.7%) compared with *apoE*$^{-/-}$ mice (98.0% ± 0.5%, $P < 0.05$, Tukey's t test following one-way ANOVA) and *apoE*$^{-/-}$*TP*$^{-/-}$ mice (98.5% ± 0.5 %, $P < 0.05$, Tukey's t test following one-way ANOVA) (Figure 2E). Compatible with these findings in the cross-sections, staining of en face preparations with silver nitrate as well as anti-PECAM revealed loss of ECs which was consistently associated with the "shoulder" of a plaque in *apoE*$^{-/-}$*IP*$^{-/-}$ mice (Figure 2F). Such EC loss was rarely seen in *apoE*$^{-/-}$ and *apoE*$^{-/-}$*TP*$^{-/-}$ mice (observations of 5 mice of each strain). Scanning electron microscopy of the aortic arch region revealed again focal endothelial disruption in the "shoulder" of atheromatous plaques of all of three *apoE*$^{-/-}$*IP*$^{-/-}$ mice examined, while none of three *apoE*$^{-/-}$ or *apoE*$^{-/-}$*TP*$^{-/-}$ mice showed such lesions. In some cases, the lesion of endothelial disruption formed a crater in which monocyte/macrophage–like cells accumulated (Figure 2G). These findings suggest that the EC loss had already occurred in vivo and was not an artifact created during sample preparation.

ICAM-1 and PECAM-1 expression on ECs in 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 overlying the lesions of the three lines 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_2 and PGI_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,

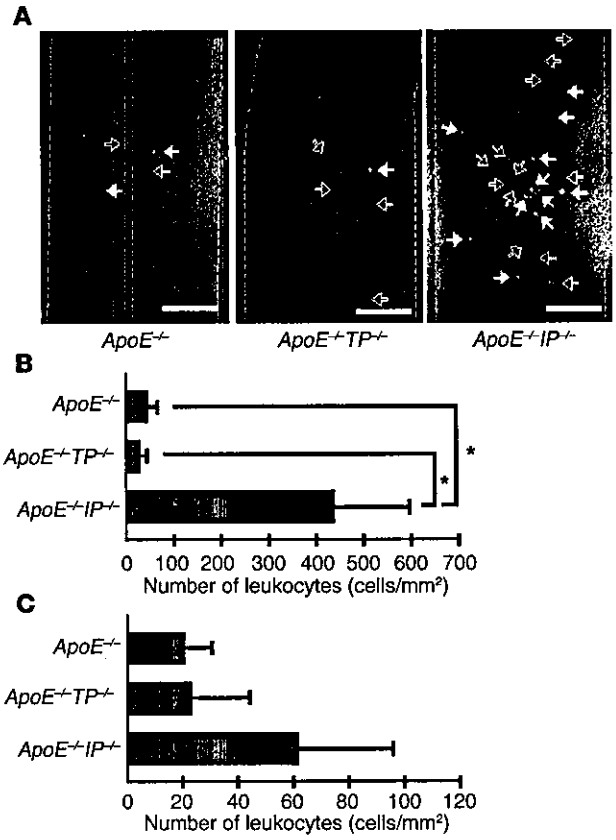

Figure 5
Intravital microscopy for leukocyte rolling and adhesion. (**A**) Fluorescence images of rolling and adherent leukocytes. Black and white arrows indicate rolling and adherent leukocytes, respectively. Vessel lumens are outlined by broken lines. Scale bars: 0.1 mm. (**B**) Quantitative analysis of rolling leukocytes. Data are means ± SEM ($n = 5$ each). *$P < 0.05$ versus *apoE*$^{-/-}$ and *apoE*$^{-/-}$*TP*$^{-/-}$ mice. (**C**) Quantitative analysis for adherent leukocytes as described in **B**.

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 with 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*$^{-/-}$*TP*$^{-/-}$ 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

TXA_2 and PGI_2 are two major prostanoids in the cardiovascular system, being abundantly produced by blood platelets and vascular endothelium, respectively. Previous studies found that TXA_2 and PGI_2 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 TXA_2 and PGI_2 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 TXA_2 and PGI_2, as seen in humans (35). Thus, the *apoE*$^{-/-}$ mouse is a suitable animal model for evaluation of the roles of TXA_2 and PGI_2 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 TXA_2 and PGI_2, 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 PGI_2 to IP is important in preventing the initiation of atherosclerosis. Impaired PGI_2 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 PGI_2 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, PGI_2 appears to exert important inhibitory actions on the initiation and progression of atherosclerosis, and the reduction in PGI_2 in the presence of normal TXA_2 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 PGI_2 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 PGI_2 under basal conditions is derived from COX-2 catalysis, both COX-1 and COX-2 contribute to the increase in PGI_2 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 PGI_2 production (15–17). In addition, TXA_2 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 TXA_2 when transformed into foam cells with modified LDL (41).

What, then, are the underlying mechanisms of the actions of TXA_2 and PGI_2 in atherogenesis? Activation and inhibition of blood platelets by TXA_2 and PGI_2, 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 TXA_2 and PGI_2. ICAM-1 expression is induced by proinflammatory cytokines from activated macrophages such as TNF-α or IL-1β (50). Signaling from PGI_2 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 TXA_2 formed in situ in atheromatous plaques acts on ECs to induce ICAM-1 expression to amplify atherogenesis. Interestingly, TXA_2 and PGI_2 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 PGI_2 and TXA_2 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 PGI_2 and TXA_2 to atherosclerosis.

Methods

Generation of apoE$^{-/-}$*TP*$^{-/-}$ *and apoE*$^{-/-}$*IP*$^{-/-}$ *double-KO mice.* *ApoE*$^{-/-}$ mice (129Ola × 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 SLC). *TP*$^{-/-}$ and *IP*$^{-/-}$ mice were then cross-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 snip samples as a template. PCR analysis was performed for apoE alleles with the sense primers exon2 (5′-GTGCTGTTGGTCACATTGCTGACA-3′) and Neo1 (5′-ATGGGATCGGCCATTGAACA-3′) for WT and mutant alleles, respectively, and the antisense primer exon3 (5′-TCAGTTCTTGTGTGACTTGGGAGC-3′); for TP alleles with the sense primers ML139 (5′-ACTTTGTTGCAGACACCACCTGTC-3′) and Neo2 (5′-TGATATTGCTGAAGAGCTTGGCGGCGA-3′) for WT and mutant alleles, respectively, and the antisense primer ML136 (5′-AAGCTTGGGTTTCAGGGACCT-3′); and for IP alleles with the sense primer CY37 (5′-GTATCTTTCAGTACCTGAGGACTG-3′) and

the antisense primers CY41 (5′-GAGCAGAAAAATTCCCAGAGGCTT-3′) and Neo17 (5′-TGACCGCTTCCTCGTGCTTTAC-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 $MgCl_2$, 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). Mice were 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 $MgCl_2$, and 5 mM KCl). Platelet-rich plasma (PRP) was prepared by centrifugation at 160 *g* for 5 minutes at room temperature. Platelet-poor plasma was obtained by further centrifugation of the blood after PRP was removed at 1,500 *g* for 10 minutes at room temperature. The number of platelets in the PRP was adjusted to 3×10^5 platelets/μl. Platelet aggregation was measured with an aggregometer (NBS Hema Tracer 601; Tokyo Koden). I-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; LDLC 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 heart and major branching arteries in the body. The primary incision followed the ventral side of the aorta and the inner curvature of the arch. To obtain a flat preparation for imaging, a second incision was made along the outer curvature of the arch. The aorta (from the heart to the iliac bifurcation) was then removed and was "pinned out" on a black wax surface in a dissecting pan using stainless steel pins 0.2 mm in diameter. After overnight fixation with the PFA solution described above and a 12-hour rinse in PBS, the aortas were briefly rinsed in 70% ethanol; immersed for 6 minutes in a filtered solution containing 0.5% Sudan IV, 35% ethanol, and 50% acetone; and destained for 5 minutes in 80% ethanol. The Sudan IV–stained aortas were photographed and were used for quantification of atherosclerotic lesions.

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.

Immunohistochemistry. 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 a 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) H_2O_2 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.

Scanning electron microscopy. The aortic trees of 20-week-old male mice were washed at 37°C for 10 minutes with PBS and were fixed at room temperature for 10 minutes with PBS containing 1% glutaraldehyde by perfusion, as described previously (69). The aortic tree was then excised, opened longitudinally, additionally fixed by immersion in PBS containing 1% glutaraldehyde at room temperature for 24 hours, dehydrated in ethanol, and processed by critical point drying with CO_2. The aortic tree specimens were then oriented with the lumens exposed, mounted with carbon paint, and coated with gold for scanning electron microscopy (T-330; Nippon Denshi).

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 FACSVantage 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 mm^2 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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動脈硬化の分子機構の解明とその成果の臨床応用

北　徹*

キーワード◉高LDL-コレステロール血症　酸化LDL　LOX-1　動脈硬化　急性冠症候群

Ⅰ はじめに

近年，生活習慣の欧米化に伴い日本人の食生活・運動習慣が激変し，それと共に生活習慣に起因した疾患の増加がみられる．ことに，動物由来の脂肪摂取過剰，カロリー過剰摂取，穀類・野菜類の摂取不足による繊維成分の不足，運動不足などが重なり，高脂血症患者，糖尿病・耐糖能異常患者，リスクファクターの集積したメタボリックシンドロームの増加が顕著である．それに伴い，虚血性心疾患（狭心症・心筋梗塞など）の罹患率の増加がみられる．これら疾患のもとになる疾患は動脈硬化である．したがって，動脈硬化の発症・進展機構が解明されれば，それに伴い治療法も開発されることになる．

1970年代に入り，Goldstein，Brown博士は，遺伝的に低比重リポ蛋白（low density lipoprotein；LDL）が上昇し，結果的にはそれに伴う動脈硬化により心筋梗塞で死亡する率が非常に高い家族性高コレステロール血症の原因を突き止め，1985年にはノーベル医学生理学賞を受賞した[1]．すなわち，LDL受容体の発見である．彼らの発見により，それまで動脈硬化研究はもっぱら栄養・脂質研究，病理学的研究が主体であったが，分子生物学的アプローチが主流になってきた．

本稿では，筆者らのグループがかかわってきた動脈硬化研究の課題，その臨床応用の成果について概説する．すなわち，①いかなるメカニズムで血中LDL値が上昇するのか，②高LDL血症と動脈硬化の架け橋は酸化LDLである，③酸化LDL受容体の単離，病変形成における意義，④急性冠症候群の診断マーカーとしての可溶型LOX-1（lectin-like oxidized LDL receptor-1）の意義などである．

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Ⅱ LDL値上昇機構

血液中を水に不溶なコレステロール・中性脂肪が運ばれ末梢組織で利用されるためには，担体（リポ蛋白）が必要である．リポ蛋白には5種類あり，それぞれカイロミクロン，超低比重リポ蛋白（VLDL），中間比重リポ蛋白（IDL），低比重リポ蛋白（LDL），高比重リポ蛋白（HDL）である．カイロミクロンは腸管で作られ食事由来の脂質を肝臓に運搬する．一方，体内で作られた脂質は肝臓でVLDLとして産生され，血中に放出され，血管壁に存在するリポ蛋白リパーゼ（LPL）で，VLDL中の中性脂肪が加水分解を受けながらIDL→LDLへと代謝を受ける．加水分解され生

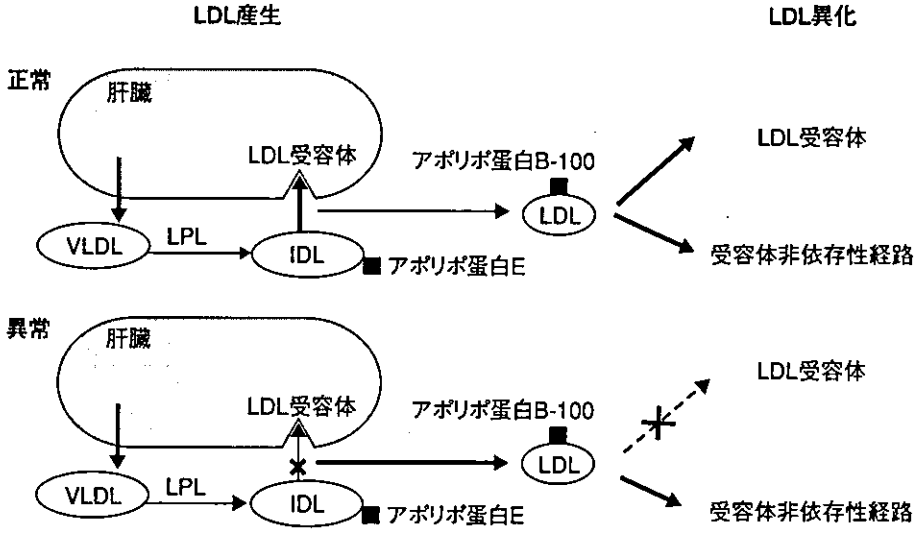

図1　LDL産生の短絡経路

LDL受容体への親和性は，アポリポ蛋白Eがアポリポ蛋白B-100より数千倍高い．
(Goldstein JL, *et al* : *N Engl J Med* 1983 ; 309 : 288−296 より引用)

じた脂肪酸は筋肉のエネルギー源として使用され，残りは脂肪組織で蓄えられる．IDLは速やかにLDLへと代謝を受けるので，血中に存在することはまれである．LDLはステロイドホルモンの原料なので，副腎，男女の性ホルモン産生組織，胆汁酸の産生組織(肝臓)，細胞増生の盛んな組織(骨髄，皮膚，精巣)で利用される．LDLは，その受け皿であるLDL受容体にその構成成分であるアポリポ蛋白B-100が認識され，細胞内に取り込まれ利用される[1]．Goldstein, Brown博士はこの事実を明らかにしたが，血液中のLDL値が制御される機序は不明であった．

筆者らは，LDL受容体欠損ウサギ（WHHLウサギ）を用いてVLDL→IDL→LDLへの代謝動態の研究の最中に，血中LDL値の制御機構を発見した．すなわち，正常では肝臓から分泌されたVLDLはIDLに代謝されると直ちにその一部は肝臓LDL受容体を介して取り込まれ，一部のIDLのみLDLへと代謝を受ける．つまり，IDLにはアポリポ蛋白B-100だけでなくEも存在する．アポリポ蛋白Eはアポリポ蛋白B-100よりもLDL受容体に数千倍親和性が高いために，LDLよりも効率良く肝臓のLDL受容体に認識され取り込まれる．一方，LDL受容体が欠損していたり半減している場合には，IDLはLDLに大量に移行するためLDLの血中濃度が上昇する[2,3]（図1）．全体として血中LDLの70%が肝臓で代謝されることが明らかになった．筆者らの成果をもとに2回目の心筋梗塞症で入院した患者に心臓移植のみならず肝臓移植を行ったところ，コレステロール値が1,000mg/dlから300mg/dlに低下したことから，ヒトにおいても肝臓LDL受容体が血中LDL値の70%を制御していることが明らかになった．

さらにWHHLウサギを用いて，HMG-CoA還元酵素阻害薬（スタチン系）と胆汁酸再吸収阻害薬の単独・併用投与が肝臓のLDL受容体mRNA，LDL受容体蛋白の誘導発現を惹起し，結果として血中LDL値の低下を引き起こすことを分子レベルで初めて明らかにした[4]．さらに，スタチン系薬剤は肝臓のアポリポ蛋白B-100の細胞内分解代謝を促進する結果，VLDLの合成抑制を起こすことを見出し，スタチン系薬剤の新たな作用機序を明らかにした[5]．現在汎用されているスタチン系薬剤，胆汁酸再吸収阻害薬の作用機序を明らかにし，高脂血症患者

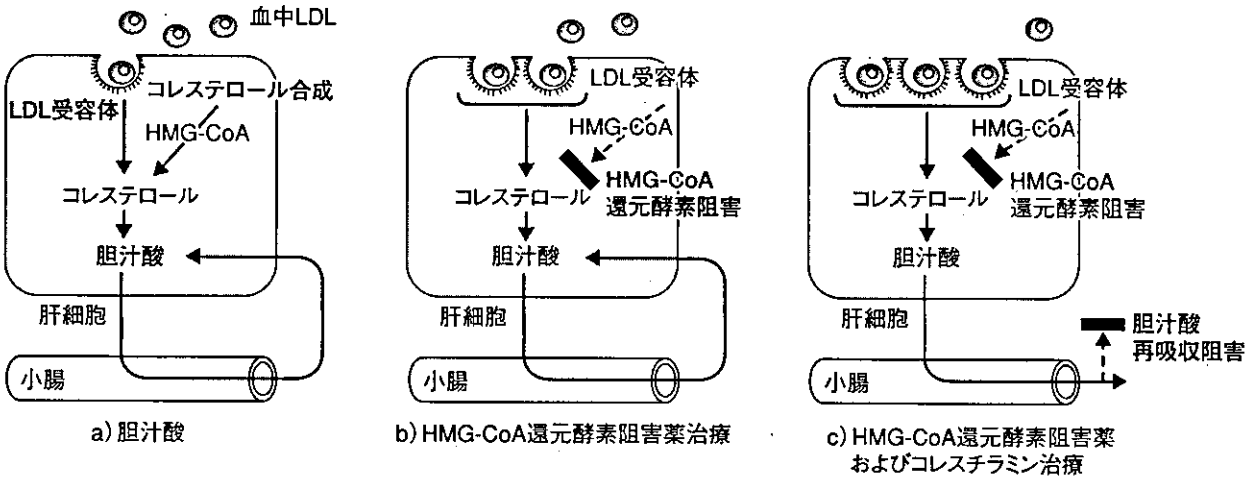

図2　肝細胞におけるコレステロール制御機構と薬剤による LDL 受容体誘導効果の模式図

の治療原理となった（図2）.

Ⅲ 高 LDL 血症と動脈硬化の架け橋は酸化 LDL である

動脈硬化の中心巣は，一部の活性 T リンパ球と単球・マクロファージ（MΦ）由来の泡沫細胞の集簇であり，いわゆる脂肪線条である．単球は血管内皮細胞に接着分子を介して結合し，走化因子 MCP-1（単球走化性蛋白 1）を介して血管内膜に侵入し，c-*fms*・M-CSF（MΦ コロニー刺激因子）系反応を介して MΦ に分化する．この事実は，動脈硬化モデルマウス（アポリポ蛋白 E 欠損マウス）に c-*fms* の機能遮断抗体を投与し，単球の MΦ への分化を抑制することにより動脈硬化の進展が抑えられたことから，筆者らによって単球の MΦ への分化が動脈硬化の進展に必須であることが明らかにできた[6]．MΦ の泡沫化には LDL の変性修飾が必須であることは，*in vitro* の研究成果から明らかにされて，なかでも LDL の酸化が重要な役割を果たしていることが筆者らや Steinburg 博士らにより明らかにされてきた[7,8]．

筆者らは，*in vivo* で WHHL ウサギに抗酸化薬プロブコールを投与することにより LDL の酸化を抑制し，結果として動脈硬化の進展を防ぐことに世界に先駆け成功し，生体内で酸化 LDL の存在を証明した．つまり，高 LDL 血症と動脈硬化をつなぐのは酸化 LDL であることを明らかにできた[7]．この仕事は 1990 年の *The Journal of NIH Research*（2：65—69）の "Research in focus" に取り上げられた．酸化 LDL は血管構成細胞（内皮細胞，平滑筋細胞，MΦ など）に作用してそれらを活性化し，接着因子・平滑筋増殖因子・マトリックスメタロプロテアーゼ・組織因子などの遺伝子発現を誘導し，動脈硬化進展を助長することが筆者らにより明らかにされた[9,10]．

Ⅳ 酸化 LDL 受容体

酸化 LDL は MΦ に受容体を介して取り込まれ泡沫化することが，Goldstein，Brown 博士によって明らかにされてきたが[11]，児玉らはその受容体の単離・構造決定に成功した．いわゆるスカベンジャー受容体である[12]．筆者らは沢村博士らと共同で，血管内皮細胞から初めて新規酸化 LDL 受容体の単離・構造決定に成功し，LOX-1 と命名した[13]．表1に LOX-1 の特徴を示す．当初は血管内皮細胞からの発現と認識していたが，その後，動脈硬化の各ステージによってその発現が異なること，つまり初期には動脈硬

表1　LOX-1の特徴・性質

1. 世界で初めて血管内皮細胞からクローニングされた酸化LDL受容体である
2. 分子量40～50kDaのII型糖蛋白で，細胞外にC型レクチン様構造をもつ
3. 動脈硬化病変の初期には主として血管内皮細胞に，成熟病変では内膜の血管平滑筋細胞やマクロファージに発現が認められる
4. 炎症性サイトカイン：腫瘍壊死因子（TNF）α，トランスフォーミング増殖因子（TGF）β，酸化LDL，アンジオテンシンII，低酸素，ずり応力で誘導される
5. 細菌，老化赤血球，アポトーシス細胞を認識する
6. 可溶型が存在する

化の血管内皮細胞に，成熟病変では血管平滑筋細胞とMΦ上に主として発現することが明らかになった[14]．各種炎症性サイトカインでの誘導，酸化LDL自体，アンジオテンシンII，ずり応力（shear stress）などで誘導されることから，動脈硬化進展に酸化LDL・LOX-1システムが大きなかかわりを有することが示唆された[15]．

LOX-1は，成熟病変では粥腫（プラーク）の内膜側，ことに肩の部分に発現がみられ，主として内膜に遊走・増殖した血管平滑筋細胞に発現が認められる．一部MΦにも発現をみる．LOX-1は動脈硬化病変の形成過程で種々の働きがあることを示唆している事実である[16]．LOX-1のもう1つの特徴は可溶型が存在することである[17]．筆者らは，島岡，米原らと共同でSR-PSOXを単離精製し，その構造決定をした[18]．SR-PSOXは主としてMΦに存在し，酸化LDLを認識する．プラークでは泡沫細胞の部分で強く染まるので，LOX-1とは異なる働きをしていることが示唆される．SR-PSOXには，Cysterらにより CXC モチーフがあることと，T細胞の走化性の役割が示唆されている[19]．さらに可溶型が存在することが明らかにされ，筆者らは血中濃度と急性冠症候群の評価のため，測定系を開発中である．可溶型LOX-1，SR-PSOXの測定系が完成し，急性冠症候群とのかかわりのさらに詳細が解明されれば，その診断に寄与することが期待される．

Ⅳ 急性冠症候群の診断マーカーとしての可溶型LOX-1

心筋梗塞の特徴は，責任冠動脈の狭窄度が50%前後が大半であることである．心筋梗塞は，プラークの破綻に伴う血栓形成を原因として起こる．したがって，プラーク破綻の機序の解明，その予知診断法の開発は急務である．

筆者らは，その手段として可溶型LOX-1に注目した．すなわち，①LOX-1は，プラークの肩の部分に主として発現しており，炎症性サイトカイン，酸化LDL，アンジオテンシンIIなどで遺伝子発現が惹起される．②成熟病変では，血管内膜に遊走した血管平滑筋細胞に主として発現している．③酸化LDL・LOX-1系は血管平滑筋細胞のアポトーシスを惹起する[16]．④可溶型LOX-1は，炎症性サイトカインでその産生が増加する[17]．以上の事実から，可溶型LOX-1の測定系を開発した．0.5ng/mlの検出限界である．非心原性疾患，狭心症，急性冠症候群で可溶型LOX-1を測定したところ，急性冠症候群で増加していることが明らかになってきた（submitted）．今まで急性冠症候群の診断マーカーとして使われてきた高感度C反応性蛋白（CRP），トロポニンTなどと比べ，その感度，特異度などで可溶型LOX-1が優れていることを明らかにしてきた[20]．現在，大規模臨床試験でその臨床的意義を検討中である．

Ⅴ おわりに

家族性高コレステロール血症の特徴は，高LDL血症と動脈硬化を基盤とした動脈硬化である．したがって，それぞれのメカニズムを研究すればLDL値の上昇機序，高LDL血症がいかなる機序で動脈硬化を引き起こすかが明らかになる．その結果，筆者らは肝臓のLDL受容体の発現量が血中LDL値を制御していること，