の速度で室温から5□まで冷却した。その後、液体窒素蒸気中に15分間静置して凍結を行った。凍結精子は液体窒素中に浸漬して保管した。

これらの実験は、佐賀大学動物実験安全管理規則ならびに佐賀大学遺伝子組換え実験安全管理規則にしたがい、関連する法令・規則（「研究機関等における動物実験等の実施に関する基本指針-文科省」、「遺伝子組換え生物の使用等の規制による生物の多様性の確保に関する法律」）を遵守して実施した。

C．研究結果

1. MMP-12 Tg WHHL MI ウサギの繁殖

自然交配での妊娠率は、80.6%（交配数31回）であった。MMP-12 Tg WHHL MI ホモ型18匹（雄10匹、雌8匹）を得ることができた。このうち、雄9匹を実験に使用するため神戸大学へ輸送した。

2. MMP-1 および MMP-9 Tg ウサギの開発

MMP-9については、2匹のファウンダーが得られており（雄1匹、雌1匹）、PCRによる遺伝子解析でF1への導入遺伝子の伝達が確認された。2匹のファウンダーから得られたF1ウサギを遺伝子の発現解析のためにそれぞれ山梨大学へ輸送した。ノーザンブロットおよびウエスタンブロットにより導入遺伝子の発現ならびにMMP-9蛋白の発現が確認された。そこで、MMP-9 Tgウサギと WHHL MI ウサギとの交配を開始した。現在までに、MMP-9 Tg WHHL MI ヘテロ型の雄2匹、雌3匹が得られている。

MMP-1についても2匹のファウンダーが得られており（雌2匹）PCRによる遺伝子解析でF1への導入遺伝子の伝達が確認された。現在、遺伝子発現解析に必要な個体数を得るための繁殖を実施している。

3. ウサギ精子の凍結保存

これまでに、MMP-12 Tg WHHL MI ウサギについては、凍結ストローで92本（融解後の精子運動率で平均30.1%）を保存した。MMP-9 Tg ウサギについては、120本（融解後の精子運動率で平均34.6%）を保存した。MMP-1 Tg ウサギについては、26本（融解後の精子運動率で平均33.8%）を保存した。

D．考察

実験動物の系統保存には、個体による維持、凍結精子もしくは凍結胚による保存方法があるが、ウサギでは射出精液の採取ならびに人工授精が容易であることから、胚の凍結保存くらべて安価で容易に実施できるため、まずは凍結精子による保存を進めた。凍結保存された精子の保存性については、これまでの我々の検討から、少なくとも5.6年間保存された凍結精子から産子が得られている。今後、さらに長期保存による影響について検討を進めてゆきたい。いっぽう、MMP-12 Tg WHHL MI ウサギの繁殖は、当初、効率的な繁殖を目的に人工授精により進めたが、期待された成績が得られなかった。しかし、人工授精から自然交配へ切り替えたことから順調に個体数を増やすことができた。凍結精子による系統保存を行なう場合、ウサギコロニーの再構築の際には、人工授精は必須の技術となることから、MMP-12 Tg WHHL MI ウサギの繁殖で人工授精が上手く行かなかった原因について検討が必要であ

ると考えられる。

E．結論

MMP-12 Tg WHHL MI ウサギの繁殖を行い実験に必要な MMP-12 Tg WHHL MI ホモ型の供給を行なうことができた。新規 MMP-9 Tg ウサギについては導入遺伝子の発現が確認できた。MMP-1 Tg ウサギについても解析に必要な匹数の F1 ウサギが得られ次第、発現解析を行い新規モデル動物としての Tg ウサギの系統確立を行う予定である。また、確立できた系統については、凍結精子による系統保存を実施した。

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G. 知的財産権の出願・登録状況

1. 特許出願

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

（別紙 4）

研究成果の刊行について

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

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Review Article

Roles of the WHHL Rabbit in Translational Research on Hypercholesterolemia and Cardiovascular Diseases

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Conquering cardiovascular diseases is one of the most important problems in human health. To overcome cardiovascular diseases, animal models have played important roles. Although the prevalence of genetically modified animals, particularly mice and rats, has contributed greatly to biomedical research, not all human diseases can be investigated in this way. In the study of cardiovascular diseases, mice and rats are inappropriate because of marked differences in lipoprotein metabolism, pathophysiological findings of atherosclerosis, and cardiac function. On the other hand, since lipoprotein metabolism and atherosclerotic lesions in rabbits closely resemble those in humans, several useful animal models for these diseases have been developed in rabbits. One of the most famous of these is the Watanabe heritable hyperlipidemic (WHHL) rabbit, which develops hypercholesterolemia and atherosclerosis spontaneously due to genetic and functional deficiencies of the low-density lipoprotein (LDL) receptor. The WHHL rabbit has been improved to develop myocardial infarction, and the new strain was designated the myocardial infarction-prone WHHL (WHHLMI) rabbit. This review summarizes the importance of selecting animal species for translational research in biomedical science, the development of WHHL and WHHLMI rabbits, their application to the development of hypocholesterolemic and/or antiatherosclerotic drugs, and future prospects regarding WHHL and WHHLMI rabbits.

1. Introduction

According to WHO, the major cause of death within member nations is cardiovascular diseases which account for about 30% of all deaths [1]. This report has indicated that cardiovascular diseases are one of the most important classes of diseases to be overcome. As main risk factors for cardiovascular diseases, hypercholesterolemia, hypertension, disorders in glucose metabolism, smoking, aging, male gender, and social stress are listed. Particularly, control of serum lipid levels is thought to be most important for the prevention of cardiovascular diseases. Currently, in the Japanese population, the upper limits of the normal ranges for serum total cholesterol and LDL cholesterol levels are 220 mg/dL and 140 mg/dL, respectively, and the lower limit of the normal range of HDL cholesterol is defined as 40 mg/dL [2]. According to studies conducted during the 1980s, the incidence of cardiovascular events increases as the serum cholesterol level increases and decreases with hypocholesterolemic treatments [3]. One potent hypocholesterolemic compound is statin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, a rate-limiting enzyme in cholesterol synthesis. The first statin (compactin) was initially developed by a Japanese pharmaceutical company, Sankyo Co. Ltd. [4], and this accelerated the development of cholesterol lowering drugs. The hypocholesterolemic effect of compactin was initially examined with rats. However, the anticipated cholesterol-lowering effect was not observed [5], and the development of this compound was ceased. On the other hand, since compactin showed a potent inhibitory effect on cholesterol synthesis *in vitro* and in chickens, researchers had been looking for other mammalian species applicable for

the assessment of this agent. They found a report of a mutant rabbit strain showing hyperlipidemia, written in a Japanese university's bulletin [6]. This rabbit strain contributed greatly to the development of this compound. The strain was the Watanabe heritable hyperlipidemic (WHHL) rabbit. This was in 1979. Currently, there are seven statins in widespread clinical use. It is estimated that statins are prescribed to more than 40 million patients worldwide and statin therapy has decreased mortality from cardiovascular diseases by 20–50% [7]. Thus statins became essential agents for the treatment of hypercholesterolemia and cardiovascular diseases. These results demonstrate the importance of selecting animal species and/or animal models for translational research to develop therapeutic agents.

This review raises the importance of selecting animal species and/or animal models for translational research by describing the history of the WHHL rabbit and its contribution to studies of hypercholesterolemia and atherosclerosis.

2. The Development of the WHHL Rabbit and Its Characteristics

The history and characteristics of the WHHL rabbit were described in a previous article [8]. In 1973, Dr. Yoshio Watanabe (1927–2008) found one male Japanese white rabbit showing hyperlipidemia. From this mutant, he established a strain, the WHHL rabbit, after seven years of selective breeding. At first, this strain was designated the hyperlipidemic rabbit (HLR) [9]. He submitted a study on this strain to an international journal and renamed it the Watanabe heritable hyperlipidemic (WHHL) rabbit [10], according to a suggestion by the editor.

The strain has 300–700 mg/dL of total cholesterol and 300–400 mg/dL of triglyceride in plasma. There were atherosclerotic lesions in the aorta and xanthoma in the digital joints. The serum glucose level and blood pressure were in normal ranges. In WHHL rabbits, the function of low-density lipoprotein (LDL) receptors on the cell membrane was almost deficient and the clearance of LDL from the circulation delayed [11]. Such symptoms closely resemble human familial hypercholesterolemia (FH), which develops spontaneously, and thus the WHHL rabbit is recognized as the first animal model of this disease. Later, the Nobel Prize winners Goldstein and Brown used WHHL rabbits to verify their hypothesis of an LDL receptor pathway for the metabolism of lipoproteins and clarified human lipoprotein metabolism [12–15]. Their studies revealed that lipoprotein metabolism in the WHHL rabbit closely resembles human FH. Consequently, WHHL rabbits were used as an animal model for the development of cholesterol-lowering agents.

One of the most important features of an animal model for hyperlipidemia is the occurrence of myocardial infarction, the final event of human hypercholesterolemia. The development of severe atherosclerotic lesions in the coronary arteries is a prerequisite for the occurrence of myocardial infarction, but the incidence of coronary atherosclerosis in the WHHL rabbit was initially very low. To establish a new strain which develops coronary atherosclerosis, serial selective breeding was conducted and in 1985, the coronary atherosclerosis-prone WHHL rabbit was developed [16]. Further, a strain with severe coronary atherosclerosis was developed in 1992 [17]. Despite such long-term efforts, the incidence of myocardial infarction remained very low. After a further seven years of selective breeding with improved criteria, such as the use of descendents of rabbits with macrophage-rich coronary lesions, a new strain of WHHL rabbits was established; the myocardial infarction-prone WHHL (WHHLMI) rabbit that spontaneously develops myocardial infarction by progression of coronary atherosclerosis followed by occlusion of the coronary arteries [18]. The characteristics of WHHLMI rabbits are described in a previous review [19]. During their establishment, marked differences in the composition of atherosclerotic plaques were found between the aorta and coronary arteries [20], and the WHHLMI rabbit became an animal model with which to examine the inhibitory effects of drugs on coronary atherosclerosis. These studies suggested genetic factors other than hypercholesterolemia to be important to myocardial infarction and coronary atherosclerosis.

Figure 1 shows the changes in serum lipid levels with aging and the distribution of cholesterol in lipoproteins among WHHLMI rabbits [8]. Serum cholesterol levels are 900–1,400 mg/dL at weaning (3 months old) and at 6 months old, and then decrease gradually (700–1,200 mg/dL at 12 months old, 600–1,100 mg/dL at 18 months old, and 500–1,000 mg/dL at 24 months old). Serum triglyceride levels are 150–500 mg/dL and the change with aging is small. The HMG Co-A reductase activity (cholesterol biosynthesis) in WHHLMI rabbits does not decrease with aging and the precise mechanism of the age-related decrease in cholesterol is still unknown [21]. About 70% of cholesterol occurs in the LDL fraction, 16% in the very low-density lipoprotein (VLDL) fraction, 13% in the intermediate density lipoprotein (IDL) fraction, and 0.8% in the high density lipoprotein (HDL) fraction. Figure 2 shows the extent of atherosclerotic lesions in the coronary arteries and aorta of WHHLMI rabbits [8]. The main coronary artery is the left circumflex artery and the atherosclerotic lesion is more progressed compared to that in the left anterior descending artery and the right coronary artery. Therefore, the degree of coronary atherosclerosis (cross-sectional narrowing) has been evaluated using the left circumflex artery. The degree of aortic atherosclerosis was shown as the ratio of the surface lesion area to the lumen surface area of the aorta. Atherosclerotic lesions develop from 2 months old. At age 12 months, coronary cross-sectional narrowing was about 80% and about 60% of the aortic lumen surface was covered by atherosclerotic lesions. At 18 months old, coronary cross-sectional narrowing and aortic lesion increased to 90% and 80%, respectively [22].

Prior to the development of the WHHLMI strain, WHHL rabbits were used to investigate mechanisms of the development of atherosclerosis, and many aspects have been clarified: accumulation of oxidized LDL in the atherosclerotic lesions [23, 24]; antiatherosclerotic effects of antioxidants (inhibition of oxidized-LDL formation) [25, 26]; the expression of monocyte adhesion molecules on arterial endothelial

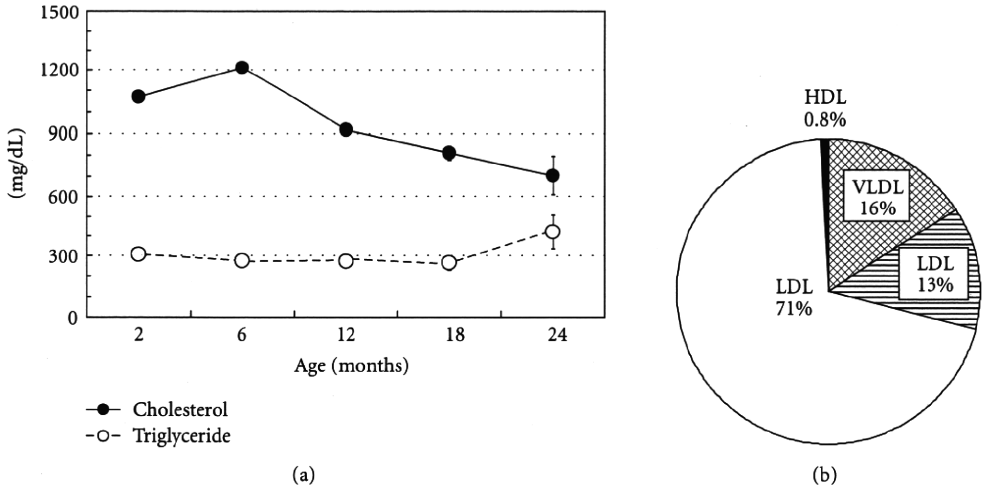

FIGURE 1: Changes in the serum lipid levels of WHHLMI rabbits with age (a), and the distribution of cholesterol in lipoproteins (b). Data are represented as the mean ± standard error of the mean. The serum cholesterol levels at 12 months old were about 900 mg/dL. Excess LDL cholesterol is atherogenic and HDL has antiatherogenic function. In WHHL rabbits, LDL is accumulated in the plasma and HDL-cholesterol is low, less than 20 mg/dL. The serum cholesterol levels decrease gradually with aging.

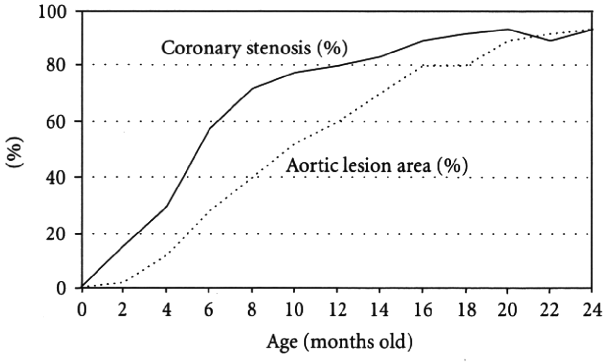

FIGURE 2: Development of atherosclerotic lesions in WHHLMI rabbits with age. The solid line denotes the degree of coronary atherosclerosis shown as coronary cross-sectional narrowing; lesion areas/area surrounded by the internal elastic lamina ×100 (%). The dotted line denotes the degree of aortic atherosclerosis; sum of the surface areas of the lesion/total surface area of the aortic lumen ×100 (%). Modified from Shiomi and Ito [8].

cells at the initiation of atherosclerosis [27]; scavenging of oxidized LDL at the lesions by macrophages through the scavenger receptors, VLDL receptors, and remnant receptors; accumulation of form cells derived from macrophages in arterial intima followed by further development of atherosclerotic lesions [28–32].

3. Species Differences in Lipid Metabolism and Atherosclerosis

As mentioned, lipoprotein metabolism in rabbits closely resembles that in humans. However, representative laboratory animals such as mice and rats have very different lipoprotein metabolism from that in humans (Table 1). Some examples of major species differences in lipid metabolism are the following. (1) In mice and rats, apoB editing enzyme is observed in the intestine and in the liver, but in humans and rabbits, this enzyme is expressed only in the intestine [33]. In humans and rabbits, apoB-48 is a major apolipoprotein of chylomicron and chylomicron remnants, which carry exogenous lipids derived from foods and apoB100 is a major apolipoprotein of VLDL, IDL, and LDL, which are endogenous lipoproteins derived from liver. In mice and rats, however, endogenous lipoproteins as well as exogenous lipoproteins also contain apoB-48, because of the expression of apoB editing enzyme in the liver [34]. Since the metabolic clearance of lipoproteins containing apoB-48 is very rapid, apoB-48 containing VLDL particles disappear rapidly from the circulation in mice and rats. As a result, the LDL lipid levels in mice and rats are very low compared with those in humans. (2) Hepatic lipase is circulating in the blood stream in mice thus different from humans in degradation of neutral lipids and transportation of free fatty acids into the tissues [35]. (3) In mice and rats, there is no cholesterol-ester transfer protein (CETP) activity in plasma, which transfers cholesterol from HDL to VLDL, IDL, and LDL [36], although CETP plays an important role in humans and rabbits. As a result, in mice and rats, the proportion of cholesterol in the HDL fraction is high compared with other lipoprotein fractions. Therefore, lipoprotein profiles of mice and rats are markedly different from that of humans, even in knockout mice lacking apoE or the LDL-receptors [8]. (4) Competitive inhibitors of a rate-limiting enzyme for cholesterol synthesis, statins, showed potent hypocholesterolemic effects in WHHL rabbits [37–45] but not in mice and rats [5]. In humans, statins are the most effective hypocholesterolemic drugs. These results demonstrate how it is important to choose appropriate species in translational research. (5) C-reactive

Table 1: Comparison of lipid metabolism, atherosclerosis, and cardiac functions between genetically modified mice and WHHLMI rabbits.

	Genetically modified mice	WHHLMI rabbits
Lipid metabolism		
Major lipoprotein in the blood	X (Chylomicron, VLDL)	O (LDL)
Structural protein in theendogenous lipoprotein	X (apoB48)	O (apoB100)
Expression of apoB editing enzyme	X (The small intestine, liver)	O (The small intestine)
CETP activity in the blood	X (No)	O (Exists)
Hepatic lipase	X (Released to circulation)	O (Bound to vessel membrane)
Atherosclerosis		
The coronary lesion	X (Resistant)	Δ (Spontaneously develops)
Composition of the lesions	X (Over accumulation of macrophages)	O (Various lesions)
VLDL receptor	X (no expression)	O (expression)
Heart		
Electrocardiogram		
Limb lead	X (Largely different waveforms)	O (Similar to humans)
Chest lead	X (Difficult to monitor)	O (Similar to humans)
Myocardial ion channel	X (I_{to} and $I_{K,slow}$)	O (I_{kr} and I_{Ks})
Myocardial fibers	X (α-myosin heavy chain)	O (β-myosin heavy chain)
Others		
Inflammatory markers	X (SAP)	O (CRP)
The hypocholesterolemic effect of statins	X (Resistant)	O (Effective)

O: similar to humans; Δ: partly similar to humans; X: largely different from humans.

protein (CRP), a major inflammatory marker in humans and rabbits, which increases in patients with acute coronary syndrome [46], is not responsive to inflammation in mice and rats, due to a lack of complement activation [47]. The major inflammatory marker of mice is serum amyloid P component (SAP), instead of CRP. (6) The types of myocardial fibers in mice are also different from those of humans and rabbits [48]. (7) Moreover, the ECG waveforms in mice and rats are clearly different from those of humans, but rabbit ECG shows similar waveforms to humans [49, 50]. As such, mice and rats have greatly different sets of factors for lipoprotein metabolism and cardiovascular diseases. Therefore, to employ mice and rats for studies on cardiovascular diseases and lipid metabolism, great care is required with analyses and/or the interpretation of the results obtained from experiments.

4. Translational Research on the Development of the Lipid-Lowering Agents

Figure 3 shows features of WHHLMI rabbits which resemble humans and applicable translational research fields. Since the WHHL rabbit is close to humans in lipoprotein metabolism, it was used for the development of various lipid-lowering agents and atherosclerosis-suppressing agents [8]. The hypolipidemic effects of various drugs have been investigated with WHHL rabbits (Table 2): cholesterol synthesis inhibitors, such as HMG-CoA reductase inhibitors and squalene synthetase inhibitors; inhibitors of microsomal triglyceride transfer protein, which works in the assembly of VLDL particles in liver; anionic exchange resins, which block the enterohepatic circulation of bile acids; omega-3 fatty acids, which are a component of fish oil; fibrates, which lower serum triglyceride levels. In studies with a cholesterol synthesis inhibitor, statin, serum total cholesterol levels of WHHL rabbits were decreased dose-dependently by 10–30% compared with the control group [37, 39]. The mechanisms for the reduction in serum cholesterol levels by statins are an increase in expression of mRNA of LDL receptors in the liver [39] and, decrease in the excretion of VLDL cholesterol from the liver in cases of high-dose treatment [38]. The agents that inhibit squalene synthetase, another rate-limiting enzyme in cholesterol synthesis, also decreased the serum cholesterol level by similar mechanisms [51]. Since a small amount of LDL receptor protein can be processed from a precursor to a mature form in WHHL fibroblasts [52], inhibition of cholesterol synthesis in the liver is expected to cause LDL receptors to accumulate on the surface of hepatocytes. Anion exchange resins absorb bile acids at the duodenum and block the enterohepatic circulation [53]. As a result, cholesterol is utilized in the hepatocytes for the synthesis of bile acids, and then the hepatocytes, which was exhausted the cholesterol pool, increase the number of LDL receptor molecules to acquire external cholesterol [39]. Therefore, the combination of an inhibitor for cholesterol synthesis and an anion exchange resin can decrease the serum cholesterol level markedly, and this was proved using WHHL rabbits [40]. Since microsomal triacylglycerol transfer protein (MTP) inhibitors are also effective in WHHL rabbits [54], they may have potential benefit for human FH. The successful treatment in WHHL rabbits means that patients with FH, excluding the LDL-receptor negative type, can be treated with these agents.

Table 2: Drug development using WHHL/WHHLMI rabbits.

	Lipid-lowering effect	Lipid-lowering effect	
		Aorta	Coronary arteries
Cholesterol synthesis inhibitors			
Statins	O	X, O	O
Squalene synthesis inhibitor	O	O	O
Anion exchanger	O	O	
Statins + Anion exchanger	O	O	O
MTP inhibitor	O		
ACAT inhibitor	X, O	X, O	X, O
Antioxidants			
Probucol	O	O	
Vitamin E	X	X, O	
Colony stimulating factor			
MCSF	X, O	O	
GMCSF	X, O	O	
Apo E	X, O	O	
Fibrate	X		
Fish oils, ommega-3 fatty acids	X, O	X, O	
Thiazolidinedione	X	Δ	Δ
Thiazolidinedione + statin	O	O	O
Antihypertensive			
ACE inhibitor	X	O	
AT-II receptor antagonists	X	O	
Calcium antagonists	X	X	
Beta-blockers	X	X	
Gene therapy	O		

O: effective; Δ: partly effective; X: no effect.
Modified from Shiomi and Ito [8].

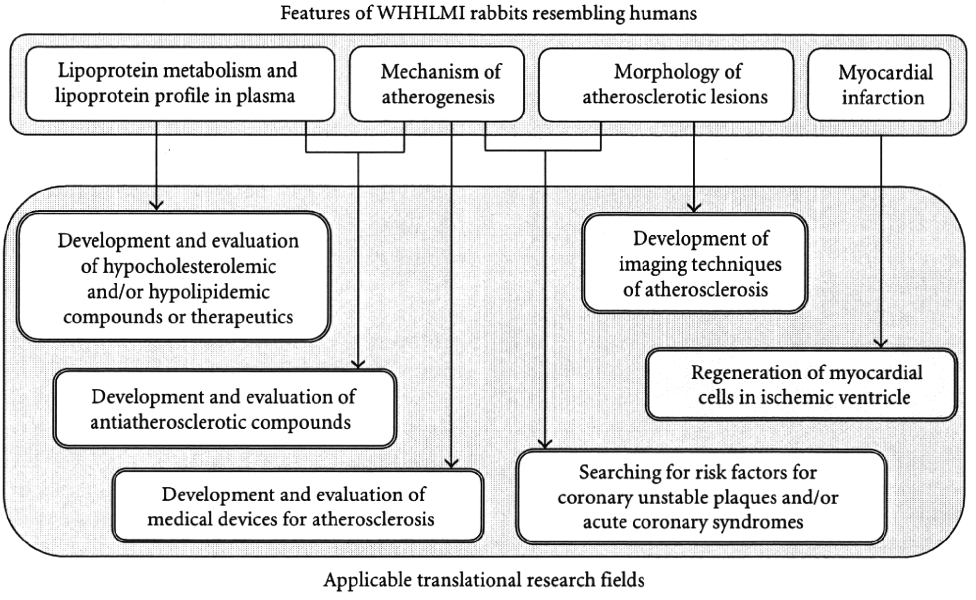

Figure 3: Features of the WHHLMI rabbit resembling humans and applicable translational research fields.

5. Translational Research on Antiatherosclerotic Effects

The purpose of lowering serum cholesterol levels is to inhibit atherogenesis and to circumvent the cardiovascular and cerebrovascular events. The WHHL rabbit contributed to prove the effects of cholesterol-lowering therapies on delaying the progression of atherosclerosis. Statin treatment resulted in a decrease in serum total cholesterol levels by 20–30%, and the cross-sectional narrowing of the coronary arteries was significantly decreased [41–45].

In several clinical studies, the incidence of cardiovascular events was significantly reduced in the statin-treated groups despite little or no improvement in coronary stenosis on evaluation by coronary angiography [55]. The WHHL rabbit contributed to the clarification of this paradoxical mechanism [42–45]. On the administration of statin to 10-month old WHHL rabbits for one year, in which coronary atherosclerosis had already developed to a mature stage, statin treatment showed not only the prevention of further progression of the coronary atherosclerotic lesions, but also various stabilizing effects on coronary plaques, such as reductions in the contents of macrophages and extra cellular lipids in lesions, and increase in the contents of collagen fibers and preservation of the smooth muscle cells in lesions. Thus it was clarified that, statin administration makes atherosclerotic lesions more stable, that is, less likely to rupture. With this study, it was confirmed that the stabilization of atherosclerotic lesions is important for the prevention of coronary events. Nowadays, more than 40 million patients worldwide are prescribed statins. Another type of cholesterol synthesis inhibitor, squalene synthesis inhibitors, that act downstream of the cholesterol synthesis pathway, also showed similar hypocholesterolemic and atheroma-stabilizing effects in WHHLMI rabbits [56].

Using WHHLMI rabbits, antiatherosclerotic effects have also been evaluated with other compounds such as omega-3 fatty acids, which decrease serum triglyceride levels by changing the composition of fatty acids [57–61]; antioxidants, such as probucol, vitamin C, and vitamin E [62–65]; agents that regulate the function of macrophages [66, 67]; drugs that inhibit the rennin-angiotensin pathway [68–71]. Interestingly, antiatherosclerotic effects of antihypertensive agents were unequal in WHHL or WHHLMI rabbits. Angiotensin converting enzyme (ACE) inhibitors and angiotensin-II receptor blockers (ARBs) showed antiatherogenic effects [69–72], but calcium antagonists and beta-blockers were not effective [73, 74]. Systolic blood pressure in WHHL and WHHLMI rabbits is 100–120 mmHg, which is slightly higher than normal [75]. This may be why calcium antagonists and beta-blockers did not show distinct antiatherosclerotic effects. In contrast, antihypertensive effects of ACE inhibitors and ARBs are mediated by suppressing the effects of angiotensin II. Angiotensin-II stimulates atherogenesis by impairing the function of arterial endothelial cells, proliferation of arterial smooth muscle cells, and inflammation [76]. These pleiotropic effects of angiotensin-II are considered to be mediated by reactive oxygen species. Thus, the WHHL rabbit is indispensable for studies on the antiatherosclerotic effects of the various compounds.

6. Imaging Technology for Evaluation of Atherosclerotic Lesions

Although it is important to evaluate drug efficacy in clinical use, it is difficult to evaluate atheroma-stabilizing effects of drugs in clinical practice. With coronary angiography, it is possible to see the degree of stenosis but difficult to evaluate the severity of lesions, if the lesions are spread and extended in the coronary arteries, or if the coronary arteries are expanded due to the outward remodeling of the vessels. Furthermore, it is very important to develop noninvasive technologies and equipment to detect dangerous lesions, that is, vulnerable plaques that are prone to rupture, not only for the diagnosis but for the prevention of cardiovascular events. As vulnerable plaques that cause cardiovascular events, soft-type plaques rich in macrophages and large lipid droplets covered with a thin fibrous cap are important. To detect such soft-type plaques, computed tomography (CT) [77], positron emission tomography (PET) [77], CT plus PET [78], magnetic resonance (MRI) [78, 79], and intravascular ultrasound (IVUS) [80] have been applied to WHHLMI rabbits. One successful example was evaluation of the antiatherosclerotic effect of probucol, a potent antioxidant, in WHHLMI rabbits by imaging with CT plus PET [81]. Ogawa et al. demonstrated clearly that imaging with CT plus PET is powerful technology to detect antiatherosclerotic effects of compounds. Once imaging technologies for the evaluation of atherosclerotic lesions are established, they can be used not only for the assessment of drug effects, but also for the detection of dangerous coronary lesions that could lead to cardiovascular events such as acute coronary syndromes and consequently the prevention of ischemic heart diseases.

7. Perspectives

To overcome cardiovascular diseases, many research issues remain unresolved, despite diligent studies for the development of diagnostic methods and lipid-lowering agents. Particularly important is clarifying the mechanism of the disruption of coronary lesions (arterial plaque rupture and the following formation of a thrombus), which depress the trigger for the onset of acute coronary syndromes, and establishment of treatments. Still no suitable animal model, which is compatible with the study of human acute coronary syndromes, has been developed. To develop a suitable animal model for human acute coronary syndromes, trial studies/experiments such as the enhancement of vulnerable coronary lesions, and application of physical pressure to coronary lesions, are currently underway with WHHLMI rabbits. To destabilize coronary lesions, serial selective breeding with new criteria such as the formation of vulnerable plaques is also ongoing, in parallel with the development of genetically modified WHHLMI rabbits overexpressing matrix metalloproteinases (MMPs), and so forth. The established strain would be a subject of analyses for the identification of

the genes/loci responsible for the phenotype established. In the near future, with advances in gene-targeting technologies by using ES or iPS cells capable of germ-line transmission, in combination with the nuclear transfer technique, more precise manipulation of the rabbit genome may also be available. Since the lesion composition and severity of coronary lesions differ even in WHHLMI rabbits, despite no difference in the serum cholesterol levels, it will be important to explore marker proteins and/or risk factors affecting coronary lesions. Once markers and risk factors relating to vulnerable coronary atheromas are found, the mechanism of cardiovascular events may be clarified. Such findings would contribute to the development of new clinical diagnostics and thence to the prevention of cardiovascular events.

In conclusion, selecting appropriate animal model is important in translational research. WHHL and WHHLMI rabbits have contributed to development of hypocholesterolemic and antiatherosclerotic compounds and medical devices, such as imaging technologies for atherosclerosis, and diagnostic techniques for acute coronary syndromes, in addition to elucidation of the mechanisms of atherogenesis and coronary plaque rupture. These studies are helpful for progression of therapeutics.

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ORIGINAL ARTICLE

Human C-reactive protein enhances thrombus formation after neointimal balloon injury in transgenic rabbits

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Summary. *Background:* High plasma levels of C-reactive protein (CRP) constitute a powerful predictive marker of cardiovascular events. Several lines of evidence suggest that CRP has prothrombogenic effects. However, whether CRP directly participates in the pathogenesis of thrombosis in vivo has not been fully clarified. *Objective:* To test whether human CRP (hCRP) affects arterial thrombus formation after balloon injury of smooth muscle cell (SMC)-rich or macrophage-rich neointima. *Methods:* We compared the susceptibility of transgenic (Tg) rabbits expressing hCRP (46.21 ± 13.85 mg L^{-1}, $n = 22$) and non-Tg rabbits to arterial thrombus formation after balloon injury of SMC-rich or macrophage-rich neointima. *Results:* Thrombus size on SMC-rich or macrophage-rich neointima was significantly increased, and was accompanied by an increase in fibrin content in hCRP-Tg rabbits, as compared with non-Tg rabbits. Thrombus size did not significantly differ between SMC-rich and macrophage-rich neointima in hCRP-Tg rabbits. Tissue factor (TF) mRNA expression and activity in these neointimal lesions were significantly increased in hCRP-Tg rabbits as compared with non-Tg rabbits. The degree of CRP deposition correlated with the elevated TF expression and thrombus size on injured neointima. In addition, hCRP isolated from hCRP-Tg rabbit plasma induced TF mRNA expression and activity in rabbit cultured vascular SMCs. *Conclusions:* These results suggest that elevated plasma hCRP levels promote thrombus formation on injured SMC-rich neointima by enhancing TF expression, but have no additive effects in macrophage-rich neointima.

Keywords: cardiovascular diseases, prognosis, smooth muscle cells, thrombosis, tissue factor.

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Introduction

C-reactive protein (CRP) is an inflammatory acute-phase reactant that has emerged as a powerful predictor of cardiovascular diseases. High levels of plasma CRP are associated with future cardiovascular events in apparently healthy individuals and with a worse prognosis in patients with acute coronary events [1].

On the other hand, CRP has also been implicated in the pathogenesis of cardiovascular diseases such as atherothrombosis. This notion was initially suggested by the demonstration that: (i) CRP is expressed in atherosclerotic lesions [2,3], where its concentration is associated with plaque instability [4]; and (ii) CRP induces proinflammatory changes in cultured vascular cells [5,6]. However, recent studies with transgenic (Tg) rabbits [7] and Tg mice [8–11] from various laboratories have indicated that CRP does not directly participate in the progression of atherosclerosis. These findings are also supported by human genetic studies [12,13], suggesting that CRP triggers cardiovascular events through other mechanisms, such as the promotion of thrombosis. This notion is supported by the following findings. Tg mice expressing CRP have impaired endothelial functions [14], and CRP reduces the expression of tissue-type plasminogen activator (t-PA) [15] and increases that of tissue factor (TF) [16] in vascular cells. In addition, injection of purified CRP activates the blood coagulation system [17]. Danenberg *et al.* [18] demonstrated that human CRP (hCRP)-Tg mice have a higher rate of thrombotic occlusion after arterial injury than non-Tg mice, whereas others have found

that hCRP does not affect the incidence of thrombus in apolipoprotein E knockout mice [19,20]. Regardless of these persistent controversies, mouse models are not apparently appropriate for examining hCRP physiologic functions, because plasma levels of CRP, even in the presence of inflammatory stimuli, are extremely low in mice as compared with humans and rabbits [21].

Recently, we established Tg rabbits expressing hCRP in the liver, and showed that the hCRP-Tg rabbit model might be useful for investigating the relationship between CRP and cardiovascular disease [7]. As in humans, but not in mice, plasma CRP in the rabbit functions as an acute reactant protein during inflammation, and hCRP can activate the rabbit complement system [7]. We previously established a rabbit model of arterial intimal injury and thrombosis [22,23], and examined whether hCRP promotes neointimal proliferation and thrombus formation in this model *in vivo*.

Materials and methods

Transgenic rabbits expressing hCRP under the control of a liver-specific promoter were generated in our laboratory as previously described [7]. Here, we studied male hCRP-Tg rabbits (2.5–3.0 kg) expressing a plasma hCRP concentration of 46.21 ± 13.85 mg L^{-1} (n = 22) and non-Tg littermates as controls (n = 25). All animal research protocols were approved by the Animal Care Committee of Miyazaki University (No. 2006-069-4), and the animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health.

Femoral artery injury models

Non-Tg rabbits and hCRP-Tg rabbits were fed with a conventional diet (non-Tg, n = 15; hCRP-Tg, n = 12) or a 0.5% cholesterol diet (non-Tg, n = 10; hCRP-Tg, n = 10) for 1 week before and 3 weeks after balloon injury to determine the effect of hCRP on the development of thrombus formation.

Neointimal lesions in the femoral artery were induced by the balloon-injury method, as previously described [22,23]. The rabbits were anesthetized with intravenous pentobarbital (25 mg kg^{-1}), and an angioplasty balloon catheter (diameter, 2.5 mm; length, 9 mm; Quantum, Boston Scientific, Galway, Ireland) was then inserted into the femoral artery under fluoroscopic guidance. The catheter was inflated to 1.5 atm and retracted by 50 mm three times to denude the endothelium. The neointimal lesions were collected for analysis 5 days and 3 weeks later (see below).

To induce thrombus formation on the neointimal surface, balloon injury was induced once again 3 weeks after the first balloon injury. A 2F balloon catheter (Baxter Healthcare, Irvine, CA, USA) was inserted via the anterior tibial artery into the femoral artery, inflated to 1.4 atm, and retracted by 30 mm three times. The rabbits were injected with intravenous heparin (500 U kg^{-1}) 15 min later, and killed with a pentobarbital overdose. Rabbits were also killed 5 days and 3 weeks after the first balloon injury, for assessment of cell proliferation and apoptosis (see Data S1). The rabbits were perfused with 50 mL of 0.01 mol L^{-1} phosphate-buffered saline (PBS) (pH 7.4) and then perfusion-fixed with 4% paraformaldehyde for histologic and immunohistochemical staining.

Blood samples were collected from the medial auditory artery into 3.8% sodium citrate (9 : 1, v/v) for evaluation of blood parameters, platelet aggregation, whole-blood coagulation and platelet adhesion under flow (see Data S1).

Histologic examinations and immunohistochemistry

The femoral arteries were fixed in 4% paraformaldehyde for 24 h at 4 °C, cut into five sections at 4-mm intervals, and embedded in paraffin. Serial sections (3-μm thick) were stained with hematoxylin and eosin (HE). Areas of neointimal lesions and thrombus size were quantified on HE-stained specimens with an image analysis system (Axio Vision 4.0.5; Carl Zeiss, Munchen, Germany) and light microscopy.

Serial sections (3-μm thick) were also immunohistochemically stained with antibodies (Abs) against hCRP, α-smooth muscle actin, rabbit macrophages, TF, rabbit fibrin, glycoprotein IIb–IIIa, and Ki-67 (Table S1). The sections were rinsed with PBS, and incubated with peroxidase-conjugated secondary Abs at room temperature for 60 min; staining was then visualized after the 3,3′-diaminobenzidine tetrahydrochloride reaction followed by counterstaining with Meyer's hematoxylin. The primary Abs were replaced with mouse non-specific IgG or sheep or guinea pig serum in control sections. Immunostained areas in whole cross-sectional areas of neointima and thrombus were quantified with a color imaging morphometry system (WinRoof, Mitani, Fukui, Japan). All quantitative analyses were performed in a blinded manner by two investigators.

Analysis of TF expression and activity

The neointima of the femoral arteries produced 3 weeks after the first balloon injury was carefully separated from the media and adventitia under a stereomicroscope. Total RNA was extracted with Trizol, and TF mRNA and β-actin expression was determined by quantitative real-time RT-PCR with SYBR Premix Ex Taq kits (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. The panel of the primers is shown in Table S2.

Rabbit plasma clotting time initiated by the neointimal homogenate was measured with a coagulation timer (Thrombotrack, AXIS-SHIELD; PoC AS, Oslo, Norway) [23,24] for evaluation of neointimal TF activity. The neointima was homogenized in Tris-buffered saline (pH 7.4) containing 5 mmol L^{-1} $CaCl_2$ and 0.1% Triton X (Nakalai Tesque, Kyoto, Japan), with a Polytron PT3000 (Kinematica, Littau, Switzerland). After centrifugation at 2500 × g for 10 min, the supernatant (vessel sample; 100 μL, containing 100 μg of

protein) was incubated for 1 min with rabbit plasma (100 μL) with or without anti-rabbit TF antibody (10 pg mL^{-1}, 4511; American Diagnostica, Stamford, CT, USA) or recombinant human TF pathway inhibitor (250 ng mL^{-1}; Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan); clotting was then initiated by adding 20 mmol L^{-1} $CaCl_2$ (100 μL). The protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL, USA). A standard curve was obtained from serial dilutions of recombinant TF (American Diagnostica), and TF activity is expressed as arbitrary units.

Expression and activity of TF mRNA in cultured vascular smooth muscle cells (SMCs)

Aortic SMCs isolated from the femoral arteries of non-Tg rabbits by the explant technique were cultured in smooth muscle growth medium (SmGM-2 bullet kit; Lonza, Basel, Switzerland) [24] for five passages. Trypsin-EDTA (Sigma, St Louis, MO, USA) was briefly added to 80–90% confluent SMCs, which were then suspended (1×10^5 cells in 100 μL of serum-free medium) in cuvettes and incubated with hCRP (20, 50 and 100 μg mL^{-1}) that was affinity-purified from hCRP-Tg rabbit plasma [7]. Total RNA was extracted, and the mRNA expression of TF and β-actin was determined as described above. The TF activity of SMCs was assessed from plasma clotting times. Briefly, rabbit plasma (100 μL) was added to viable SMCs (1×10^5 cells in 100 μL of serum-free medium) in cuvettes for analysis with a coagulation timer (AXIS-SHIELD), and clotting assays were performed as described above.

Statistical analysis

All data are presented as means ± standard deviations. Differences for individual groups were tested with Student's *t*-test and ANOVA (GraphPad Prism version 4.03; GraphPad Software, San Diego, CA, USA). Three groups of TF mRNAs and activities were analyzed by one-way ANOVA followed by Bonferroni's multiple-comparison test in the cell culture study. Platelet adhesion was analyzed in the flow chamber system by two-way ANOVA. Microscopic assessments of femoral arteries, TF mRNA and activity of neointima, blood parameters, platelet aggregation and thromboelastogram assay findings between two groups were compared with Student's *t*-test. Relationships between factors were evaluated with linear regression analysis. Statistical significance was established at $P < 0.05$.

Results

Effects of hCRP on the neointimal lesions

We initially investigated the effects of hCRP on the neointimal lesions of the femoral arteries induced by balloon injury. Figure 1 shows that neointimal lesions at 3 weeks after injury contained mainly SMCs with a few macrophages in rabbits fed

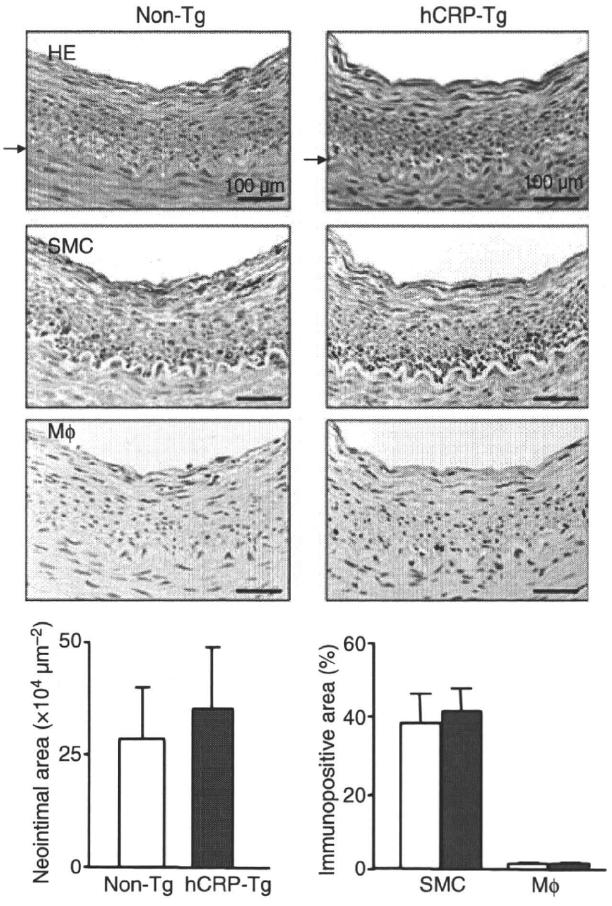

Fig. 1. Neointimal formation 3 weeks after balloon injury. Neointimal lesions mainly contain smooth muscle cells (SMCs) with a few macrophages (Mφ). Neointimal area and cellular components (SMCs and macrophages) do not significantly differ between transgenic (Tg) and non-Tg rabbits ($n = 6$ per group). Arrowheads indicate internal elastic lamina. hCRP, human C-reactive protein; HE, hematoxylin and eosin.

with a conventional diet, but neointimal size and cellular components (both SMCs and macrophages) did not significantly differ between Tg and non-Tg rabbits. Neither cell proliferation nor apoptosis differed significantly in the neointima of Tg and non-Tg rabbits at 5 days and at 3 weeks after balloon injury (Fig. S1). Immunohistochemical staining frequently revealed hCRP-immunoreactive proteins in the neointimal lesions (but not in the normal intima; data not shown) of hCRP-Tg rabbits (Fig. 2). RT-PCR analysis did not detect any hCRP mRNA transcripts in the neointimal lesions (Fig. S2), suggesting that hCRP-immunoreactive proteins were derived from the circulation.

We immunohistochemically stained the neointima to determine whether hCRP affects TF expression in neointimal SMCs. We found that the level of immunoreactive TF proteins was increased three-fold in hCRP-Tg rabbits as compared with non-Tg rabbits (Fig. 2). Furthermore, TF mRNA expression and activities were increased 1.8-fold and 4.5-fold, respectively, in the neointimal lesions of Tg rabbits as compared with non-Tg rabbits (Fig. 3). Taken together, these data indicated that

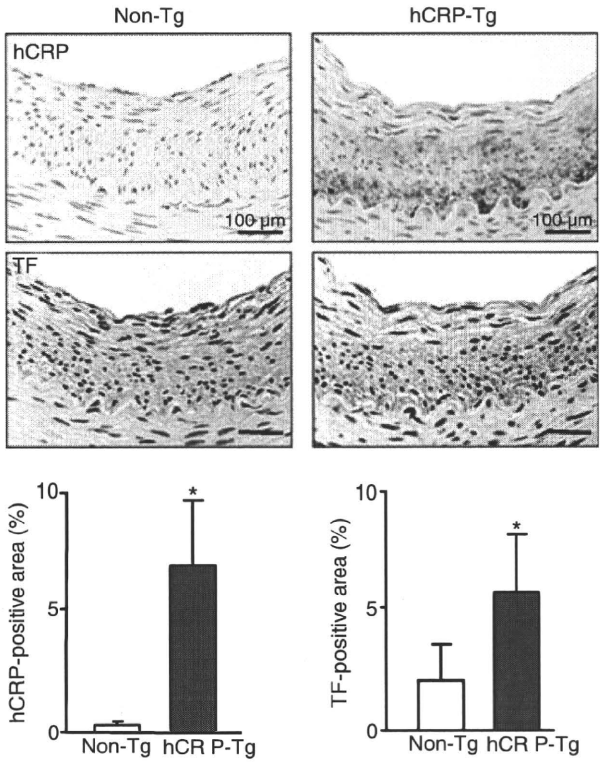

Fig. 2. Immunohistochemical demonstration of C-reactive protein (CRP) and tissue factor (TF) in neointima. Human CRP (hCRP)-immunoreactive protein is frequently present in neointima of hCRP-transgenic (Tg) rabbits, whereas TF-immunoreactive protein is present in that of both Tg and non-Tg rabbits. The positive area was quantified with an image analysis system, as described in Materials and methods (n = 6 per group). *P < 0.01 vs. non-Tg rabbits.

hCRP in neointimal lesions is associated with, or induces, TF expression but does not affect the SMC proliferation induced by balloon injury in hCRP-Tg rabbits.

Expression/activity of TF is induced by hCRP in cultured vascular SMCs

To determine whether hCRP can stimulate the TF expression by SMCs shown in hCRP-Tg rabbits, we investigated the effects of hCRP isolated from Tg rabbit plasma on the expression of TF in cultured rabbit SMCs. The expression of TF mRNA was significantly increased after incubation with hCRP (50 and 100 mg L^{-1}) for 1 h, but this effect disappeared after 6 h (Fig. 4, left). In contrast, TF protein activity induced by hCRP incubation was significantly increased by 6 h (Fig. 4, right).

Increased platelet–fibrin thrombus formation

We postulated that the pathologic procoagulant state of the neointima of hCRP-Tg rabbits would lead to enhanced thrombogenesis, and therefore compared thrombus formation induced by a second balloon injury on the neointima of the femoral arteries. Thrombi appeared on the injured neointima at 15 min after the injury, and homogeneously covered the damaged neointimal surface (Fig. 5A). Immunohistochemical staining showed that the neointimal thrombi consisted of aggregated platelets and fibrin in both non-Tg and Tg rabbits. Quantitative analysis revealed that thrombus areas and fibrin concentrations were increased 2.5-fold and 1.5-fold, respectively, in Tg rabbits as compared with non-Tg rabbits (Fig. 5C). The areas of CRP immunopositivity significantly correlated with areas of TF positivity and thrombus. The TF-positive area significantly correlated with thrombus and fibrin areas (Fig. 6A–D). To exclude the possibility that hCRP affects platelet aggregation, adhesion and whole-blood hemostatic parameters that could also lead to increased thrombus formation in Tg rabbits, we measured these parameters along with blood counts and prothrombin time (PT)/activated partial thromboplastin time (APTT) in non-Tg and Tg rabbits. However, they did not significantly differ (Fig. S3 and Table S3).

Neointima and thrombus formation in hyperlipidemic rabbits

Neointima and thrombus were induced in hCRP-Tg and non-Tg rabbits fed with a 0.5% cholesterol diet, to determine whether hCRP can stimulate TF expression in macrophage-rich neointima and increase thrombus formation. The macrophage content was significantly increased in the neointima of both types of rabbit fed with a cholesterol diet as compared with a conventional diet. However, neointimal size and cellular components did not significantly differ between non-Tg and hCRP-Tg rabbits (neointimal area, non-Tg 35.9×10^4 μm^2, hCRP-Tg 37.2×10^4 μm^2, n = 5 each; SMCs, non-Tg 40.2%, hCRP-Tg 45.8%; macrophages, non-Tg 13.0%, hCRP-Tg 14.4%). Immunohistochemical staining revealed hCRP-immunoreactive proteins in the macrophage-rich neointimal lesions. TF mRNA expression and activities in the neointima and thrombus 15 min after the second neointimal balloon injury were significantly increased in Tg rabbits as compared with non-Tg rabbits (Fig. S4A,Band Fig. 5B). However, thrombus size did not significantly differ between SMC-rich and macrophage-rich neointima in hCRP-Tg rabbits (Fig. 5C). Whole-blood cell counts, platelet function and blood coagulation parameters did not significantly differ between non-Tg and Tg rabbits (Table S4, Fig. S4C–E).

Discussion

We investigated the effect of hCRP on neointimal proliferation and thrombus formation on injured neointima after balloon injury in hCRP-Tg rabbits. The average plasma level of hCRP in Tg rabbits was 46 mg L^{-1}, which was in agreement with a study of interactions between CRP and vascular pathophysiology in humans with high CRP levels. A recent large-scale clinical study showed that the crude relative risk of cardiovascular diseases increases eight-fold among individuals with levels of CRP > 20 mg L^{-1} [25].

In the neointimal lesions of Tg rabbits, hCRP-immunoreactive proteins were frequently detected around SMCs and

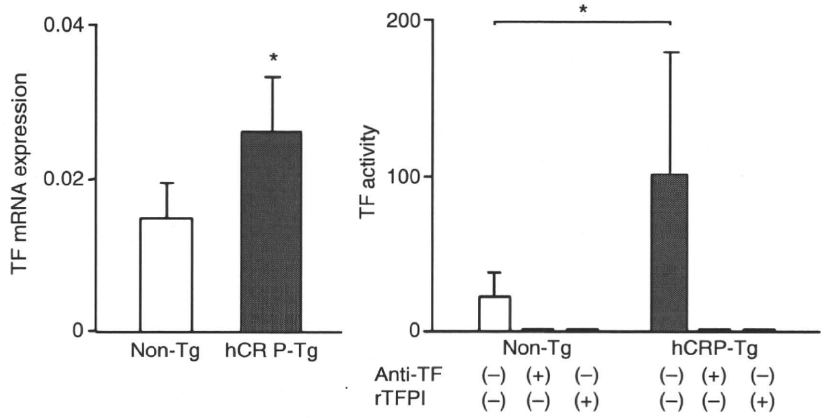

Fig. 3. Tissue factor (TF) mRNA expression and activity in neointima of femoral arteries. TF mRNA expression was analyzed by real-time RT-PCR, and is expressed as TF/β-actin ratios. The TF activity of neointima is 1.8-fold and 4.5-fold higher in terms of mRNA expression and activity, respectively, in transgenic (Tg) rabbits than in non-Tg rabbits. Anti-rabbit TF antibody (10 pg mL^{-1}, < 0.5 arbitrary units) or recombinant TF pathway inhibitor (rTFPI) (250 ng mL^{-1}, < 0.6 arbitrary units) completely blocked increased TF activity in Tg rabbits (n = 6 each; *P < 0.01 vs. non-Tg rabbits). hCRP, human C-reactive protein.

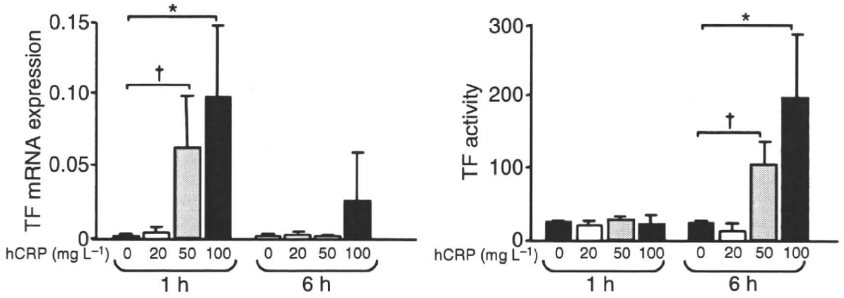

Fig. 4. Tissue factor (TF) mRNA expression and activity in cultured vascular smooth muscle cells (SMCs). TF mRNA expression and activity are expressed as TF/β-actin ratios and arbitrary units, respectively. Vascular SMCs isolated from normal rabbit femoral arteries were incubated with human C-reactive protein (hCRP) (20, 50 and 100 mg L^{-1}) purified from hCRP-transgenic (Tg) rabbit plasma for 1 or 6 h. TF mRNA expression was significantly increased after incubation with 50 and 100 mg L^{-1} hCRP for 1 h, but this effect disappeared after 6 h, whereas TF protein activity was significantly increased at 6 h. Anti-rabbit TF antibody (10 pg mL^{-1}) or recombinant TF pathway inhibitor (250 ng mL^{-1}) (data not shown) (n = 6 each) blocked this increase in activity caused by 100 mg L^{-1} hCRP. *P < 0.01, †P < 0.05 vs. control).

macrophages in the femoral arteries, which is similar to CRP deposition in the atherosclerotic lesions of aortas and coronary arteries in Tg rabbits fed with a cholesterol-rich diet [7]. Despite such obvious CRP deposition in the lesions, we did not find any differences in vascular cell proliferation and apoptosis between the two groups, suggesting that CRP is not involved in the enhanced SMC proliferation and macrophage infiltration in vascular lesions. Although these findings contradict those of previous studies [26,27], they support the notion that CRP in the lesions is not atherogenic [7–11].

Because the primary sources of TF in the non-diseased vascular wall are SMCs, and TF expression was increased remarkably after injury [28,29], we compared TF expression in neointimal lesions of Tg and non-Tg rabbits. We found that both TF-immunoreactive proteins and mRNA expression activity were significantly increased, along with TF clotting, in the neointima of Tg rabbits as compared with non-Tg rabbits, suggesting that hCRP upregulates TF expression in neointimal SMCs. These findings are further supported by the observation that incubation of cultured SMCs with hCRP induces TF expression in vitro, which is consistent with other findings [30]. The induction of TF expression in SMCs by CRP is mediated by FcγRIII (CD16), a cell surface IgG Fc receptor, the p44/42 MAPK pathway, and the generation of reactive oxygen species [26]. It should be noted that TF activities in the uninjured aortas of both hCRP-Tg and non-Tg rabbits did not differ significantly (data not shown), indicating that local CRP deposition in the neointimal lesions is necessary for TF upregulation. This finding contrasts with the study by Wu *et al.* [16], who found that SMCs isolated from the normal aortas of CRP-Tg mice express more TF and TF activities than non-Tg mice. The discrepancy might be attributable to species differences in the biological effects of hCRP on vascular SMCs.

Because TF is a critical factor in the initiation of blood clotting, increased TF expression in the arterial wall might trigger thrombosis after injury such as plaque rupture. Our study showed that the size of thrombi formed on the neointimal surface of hCRP-Tg rabbits was significantly increased as