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Suppression of neointimal thickening by a newly developed HMG-CoA reductase inhibitor, BAYw6228, and its inhibitory effect on vascular smooth muscle cell growth

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1 The aim of this study was to determine whether BAYw6228 (BAYw), a newly developed 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, could suppress an atherogenic process such as intimal thickening by a mechanism other than lowering the level of serum cholesterol.

2 First, we evaluated the *in vitro* effect of BAYw on the proliferation of vascular smooth muscle cells (SMC) from various species: Sprague-Dawley (SD) rats, New Zealand (NZ) white rabbits, intimal cells from Watanabe hereditary hyperlipidemic (WHHL) rabbit and SMC from the new-born human aorta. The increasing rate of total protein content of these cells was inhibited by the addition of BAYw in a dose-dependent fashion. In the presence of 2% foetal calf serum (FCS), the value of IC_{50} was 1.0 μM in SD rats. 2.1 μM in NZ white rabbits, and 0.3 μM in WHHL rabbits. With human SMC, the value was 0.02 μM in the presence of 10% FCS and 0.2 μM with a mixture of growth factors.

3 Based on these above *in vitro* findings, we next examined the *in vivo* effect of the agent to determine whether it could suppress rabbit intimal thickening induced by balloon catheterization. A balloon catheter was inserted from a peripheral branch of the left external carotid artery to the aorta to denude the endothelium of the left common carotid artery in Japanese white rabbits. After 12 days they were divided into control and BAYw groups. The former were subcutaneously injected with saline and the latter with BAYw 1 mg kg^{-1} day^{-1}. Two days after the beginning of treatment, a second balloon injury was performed to the previously injured left common carotid artery in both groups. After another two weeks, the left common carotid artery was removed and variously stained. Although the total serum cholesterol in the BAYw group was significantly lower than in the control ($P<0.05$), the difference was not enough to affect intimal thickening. In addition, the BAYw group had a smaller intima/media ratio than the control group, decreasing to 45% of control ($P<0.05$). By anti-α smooth muscle actin antibody staining, these intimal thickening areas were entirely occupied by SMCs, and their amount was attenuated by BAYw. By anti-rabbit macrophage antibody (RAM 11) staining, the number of positive cells in the intimal thickening was markedly decreased in the BAYw group compared to control ($P<0.01$).

4 These results indicate that BAYw has an inhibitory effect on intimal thickening by attenuating intimal SMC proliferation and infiltration of macrophages, suggesting that BAYw could be effective in the prevention of the progression of atherosclerotic plaque-like restenosis after angioplasty.

Keywords: BAYw6228; HMG-CoA reductase Inhibitor; smooth muscle cell; macrophage; balloon catheterization; intimal thickening; percutaneous transluminal coronary angioplasty (PTCA)

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a modern treatment regime of certain coronary diseases. However, restenosis after successful PTCA is a serious problem that limits the long-term efficacy of this procedure, occurring in 20–50% of patients (Faxon & Currier, 1995). The mechanism of restenosis remains unclear, but it is generally considered that the denuded intima is exposed to circulating blood elements and that immediate deposition and aggregation of platelets occurs after PTCA (Wilentz *et al*., 1991). This occurs together with proliferation and migration of vascular smooth muscle cells (SMCs) in the neointima, playing an important role in the progression of atherosclerotic plaque and the subsequent restenosis of coronary arteries (Popma *et al*., 1991; Liu *et al*., 1994).

Recent studies have shown that 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, simvastatin and lovastatin, can reduce the severity of arterial lesions and cardiovascular diseases in addition to decreasing the levels of plasma total cholesterol (T-Cho) and low density lipoprotein (LDL) in animals (Kobayashi *et al*., 1989; Zhu *et al*., 1992; Otto *et al*., 1995) and man (Kane *et al*., 1990; Mack *et al*., 1992). The mechanism by which these drugs inhibit the proliferation and migration of SMCs in the early stages of atherosclerosis is considered to be by decreasing intracellular synthesis of mevalonate. Bocan *et al*. (1994) showed that a marked reduction in atherosclerotic lesion size with treatment by HMG-CoA reductase inhibitors primarily resulted from a decrease in non-macrophage components like SMCs and extracellular matrix. However, their experiments were performed with a single balloon catheterization and the effect was evaluated in connection with improvement of hyperlipidaemia.

We believe that more extensive and detailed studies of the antiatherosclerotic effect of HMG-CoA reductase inhibitors

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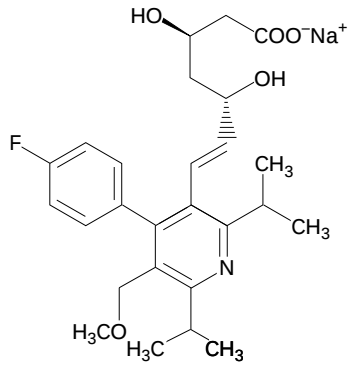

Figure 1 Chemical structure of BAYw6228.

are called for. Therefore, we attempted to determine whether BAYw6228 (BAYw), a newly developed HMG-CoA reductase inhibitor (Figure 1), might be efficient at inhibiting the proliferation of SMCs in various species *in vitro*, and at suppressing intimal thickening *in vivo*. We first measured the total protein content of SMCs and then performed two balloon catheterizations on normal rabbits before and during the administration of the agent.

Animal SMC cultures and proliferation assay

In order to obtain SMCs from the aorta of Sprague-Dawley (SC) rats, of New Zealand (NZ) white rabbits and the intima from Watanabe heritable hyperlipidemic (WHHL) rabbits, the explant technique was performed, and SMCs were subcultured with MEM supplemented with 10% FCS as described previously (Fischer-Dzoga *et al*., 1973). After reaching confluence, these SMCs were trypsinized and plated on 48 multiwell dishes at a density of 10,000 cells cm^{-2} in SD rat, and 4,000 cells cm^{-2} in both NZ white and WHHL rabbits. They were cultured with the above medium for 24 h, then maintained to reach quiescence in serum-free MEM for 72 h. After this period, MEM supplemented with 2% FCS and various amounts of BAYw (0, 0.01, 0.03, 0.1, 0.3, 1 or 3 μM) were replaced for another 48 h.

Human SMC culture and proliferation assay

Human SMCs from the new-born infant aorta were plated at a density of 8,000 cells cm^{-2} on 12 multiwell dishes. They were cultured with SmGM supplemented with 5% FCS for 24 h, then maintained to reach quiescence in serum-free MCDB131 for 24 h. After this period, MCDB131 supplemented with 10% FCS and various concentrations of BAYw (0, 0.001, 0.006, 0.01, 0.03, 0.1, 0.3, 1 or 3 μM) was used as incubation medium for another 48 h. In addition, SmGM supplemented with 5% FCS and the above concentrations of BAYw were also incubated with the cells for the same duration.

Measurement of protein content and inhibition of SMC growth

The cells were washed with PBS and then solubilized with 0.1 N NaOH. Their protein content was determined by the method of Lowry *et al*. (1951). The increase in protein content from the pretreatment stage (seeded) to the control (proliferated) was assumed to be 100% cell proliferation, and the % inhibition of SMC growth by BAYw was calculated as follows:

$$\%\ \text{inhibition} = 100 - 100 \times [(\text{PC treated} - \text{PC pre})/(\text{Pc control} - \text{PC pre})]$$

where, PC treated: the value of protein content (μg/well) in the cells cultured for 48 h with addition of BAYw; PC pre: the value of protein content (μg/well) in the cells before culture; PC control: the value of protein content (μg/well) in the cells cultured for 48 h without addition of BAYw.

Balloon catherization into the left common carotid artery of rabbit

Male Japanese white rabbits weighing approximately 3.5 kg were used. They were maintained on laboratory chow diet (Clea Japan Inc., Tokyo, Japan) and water *ad libitum* for the experimental course. In each rabbit, under chloral hydrate anaesthesia (30 mg kg^{-1}), the left common carotid artery was exposed through an incision in the neck. After a minor incision in this artery had been made, a 2F Forgaty catheter was inserted from a peripheral branch of the left external carotid artery to the aorta, care was taken not to interrupt the blood flow to the left main branch of the external and internal carotid arteries. The inside space of the balloon was filled with water, and intraluminal passages with 0.04 ml of water were done three times to denude the artery of endothelium. After this treatment, the branch used for the catheter insertion was ligated and the skin incision closed. On day 12 after the procedure, the rabbits were divided into control and BAYw groups, the former were subcutaneously injected with saline, and the latter with 1 mg kg^{-1} BAYw solubilized in water. These injections were given daily until the end of this experiment. On day 15, in all the rabbits, the second balloon catheterization was performed in order to produce further severe intimal thickening through the left common carotid artery. A 2F Forgaty catheter was inserted from another peripheral branch of the left external carotid artery to the aorta under chloral hydrate anaesthesia (30 mg kg^{-1}). Intraluminal passages with 0.03 ml of water were done three times through the artery. Finally, on day 28 after the first balloon injury, after overnight starvation, the animals were killed and the extent of neointimal formation in the left common carotid artery was examined.

Histological analysis and measurement of DNA content in the left common carotid artery

The left common carotid arteries were rapidly removed and divided into five cross-sectional pieces at 5 mm intervals. Four pieces, except for the middle one, were fixed with 4% paraformaldehyde for six hours, and these tissues were subsequently processed for paraffin embedding. Cross-sections were stained with Elastica Masson (EM), and immunohistochemical staining was with SMA or RAM11 by the ABC method (Hsu *et al* 1981). The areas of the intima, measured from the internal elastic lamina to the luminal surface, and the medial layer in each section of the left common carotid artery were calculated by a microscopical image analysing system, XL-10 (OLYMPUS, Tokyo, Japan). The value of the intimal area to medial area was the area ratio of intima/media layer (I / M ratio). As for the ABC method, the sections were deparaffinized and endogenous peroxidase was quenched by a 15 min incubation with 0.3% H_2O_2. The sections were then incubated with a 1:2000 dilution of SMA or a 1:50 dilution of RAM11 overnight at room temperature. Bound antibody was visualized by the ABC method with 3,3′-diaminobenzidine tetrahydrochloride-$NiCl_2$ as chromogen. The number of cells positive for RAM11 in the neointima was counted under X100 magnification in each preparation.

The middle pieces of the left common carotid arteries were used for measurement of DNA content. Briefly, they were incubated at 55°C for 24 h in Tris buffer (50 mM Tris, 100 mM EDTA) containing 0.5 mg ml^{-1} proteinase K; 2 ml of sodium phosphate buffer was added and the solution mixed with Hoechst dye 33258. Thereafter their fluorescences were measured to determine the DNA content of the tissues.

Serum lipid measurement

Blood samples were taken from the inferior vena cava after the rabbits had been starved overnight, and serum was obtained by centrifugation for measurement of T-Cho, triglyceride (TG) and HDL-cholesterol (HDL-C). T-Cho and TG levels were

determined by an enzymatic method, and HDL-C by precipitation (a modified phosphotungstate-$MgCl_2$ method).

Methods

Materials

BAYw was supplied by Bayer Yakuhin, LTD. (Osaka, Japan). Minimum essential medium (MEM) was purchased from GIBCO BRL (Grand Island, NY, U.S.A.), foetal calf serum (FCS) from Whittaker Bioproducts (Walkersville, MD, U.S.A.), MCDB 131 and SmGM consisted of MCDB 131 containing 10 ng ml^{-1} human recombinant epidermal growth factor and 0.39 μg ml^{-1} dexamethasone from Sanko Junyaku Co. Ltd. (Tokyo, Japan), culture plates and flasks from Iwaki Glass Co. Ltd. (Funabashi, Japan), SMCs from the new-born infant aorta from KURABO (Osaka, Japan), a 2F Forgaty catheter from Baxter Healthcare (Santa Ana, CA, U.S.A.), anti-mouse α-smooth muscle actin antibody (SMA) from Sigma Chemical Co. (St. Louis, MO, U.S.A.), anti-rabbit macrophage antibody (RAM11) from Dako A/S (Glostrup, Denmark), avidin-biotin-peroxidase complex (ABC) kit from Vectastains (Vector Laboratories, CA, U.S.A.), and Hoechst dye 33258 from Hoechst Japan (Tokyo, Japan).

Statistical analysis

The mean value obtained for each preparation from one rabbit was calculated as one datum. The results are expressed as mean ± s.e.mean. The Mann-Whitney U test or non-paired Student's t test was used for comparisons, and $P<0.05$ was considered to be statistically significant.

Results

SMC growth in SD rat, NZ white and WHHL rabbits

After the stimulation by 2% FCS for 48 h, the protein content in the absence of BAYw increased approximately two fold in SD rats and NZ white rabbits, and three fold in WHHL rabbits compared to those from the pretreatment state (Tables 1, 2 and 3, respectively). BAYw inhibited the SMC growth in both SD rats and NZ rabbits, the IC_{50} was 1.0 μM in SD rats and 2.1 μM in NZ rabbits. In WHHL rabbits, surprisingly, the IC_{50} was one-seventh (approximately 0.3 μM) of that in NZ rabbits, indicating that intimal SMCs from atheromatous plaque have a higher sensitivity to BAYw compared to those from normal media.

Human SMC growth

In human SMC, the stimulation with 10% FCS or the mixture growth factors, in the absence of BAYw, increased the protein content approximately four fold compared to the pretreatment values (Tables 4 and 5). BAYw inhibited the SMC growth induced by these stimuli, but there was an approximately ten fold difference between the IC_{50} values with the different stimuli (10% FCS, 0.2 μM; the mixture of growth factors, 0.02 μM).

Body weights and serum lipids of rabbits after balloon catheterization

No rabbit died in the two groups during the experimental procedures. BAYw did not significantly modify the body weight (control, 3.65 ± 0.04 kg ($n=7$); BAYw-treated 3.62 ± 0.09 kg ($n=5$). The serum levels of T-Cho, TG and HDL-C are shown in Table 6. T-Cho in the BAYw group was significantly lower than in the control (BAYw, 16.10 ± 0.92 ($n=5$); control, 21.57 ± 2.05 ($n=7$), $P<0.05$). There were no differences in the levels of TG and HDL-C between the two groups.

Table 1 Effect of BAYw6228 on proliferation of rat smooth muscle cells

		Protein (mg/well)	*Inhibition* (%)
Pretreatment		7.18 ± 0.14	
Control		14.72 ± 2.22	0.0
BAYw6228 (μM)	0.03	14.98 ± 0.70	−3.4
	0.1	16.58 ± 0.51	−24.7
	0.3	14.36 ± 0.27	4.8
	1	11.67 ± 0.36	40.5
	3	7.18 ± 0.10	100.0

Values represent mean ± s.e.mean of three experiments, and statistical significance was assessed by non-paired Student's t test.

Table 2 Effect of BAYw6228 on proliferation of rabbit smooth muscle cells

		Protein (mg/well)	*Inhibition* (%)
Pretreatment		3.15 ± 0.14	
Control		5.89 ± 0.38	0.0
BAYw6228 (μM)	0.01	5.89 ± 0.14	0.0
	0.3	5.32 ± 0.18	20.8
	1	5.27 ± 0.37	22.6
	3	4.13 ± 0.16	64.2

Values represent mean ± s.e.mean of three experiments, and statistical significance was assessed by non-paired Student's t test.

Table 3 Effect of BAYw6228 on proliferation of WHHL rabbit intimal cells

		Protein (mg/well)	*Inhibition* (%)
Pretreatment		5.35 ± 0.31	
Control		14.30 ± 0.38	0.0
BAYw6228 (μM)	0.03	12.54 ± 1.29	19.7
	0.1	12.14 ± 0.10	24.1
	0.3	10.13 ± 0.51	46.6
	1	7.01 + 0.58	81.5
	3	4.50 ± 0.09	109.5
	10	4.80 ± 0.30	106.1

Values represent mean ± s.e.mean of three experiments, and statistical significance was assessed by non-paired Student's t test.

Table 4 Effect of BAYw6228 on proliferation of human smooth muscle cells induced by 10% FCS

		Protein (mg/well)	*Inhibition* (%)
Pretreatment		0.80 ± 0.28	
Control		3.31 ± 0.29	0.0
BAYw6228 (μM)	0.001	3.75 ± 0.35	−17.5
	0.006	3.67 ± 0.14	−14.3
	0.01	4.70 ± 0.64	−55.4
	0.03	3.98 ± 0.21	−26.7
	0.1	2.87 ± 0.29	17.5
	0.3	1.75 ± 0.00	62.2
	1	0.08 ± 0.00	128.7
	3	0.08 ± 0.00	128.7

Values represent mean ± s.e.mean of three experiments, and statistical significance was assessed by non-paired Student's t test.

Table 5 Effect of BAYw6228 on proliferation of human smooth muscle cells induced by a mixture of growth factors

		Protein (mg/well)	*Inhibition* (%)
Pretreatment		1.01±0.28	
Control		3.59±0.34	0.0
BAYw6228 (μM)	0.001	3.43±0.24	6.2
	0.006	3.19±0.20	15.5
	0.01	2.39±0.35	46.5
	0.03	2.39+0.21	46.5
	0.1	0.88±0.16	105.0
	0.3	0.08+0.00	136.0
	1	0.16+0.08	132.9
	3	0.64+0.16	114.3

Values represent mean±s.e.mean of three experiments, and statistical significance was assessed by non-paired Student's *t* test.

Table 6 Serum lipid concentration of experimental rabbits

	Control group ($n=7$)	*BAYw group* ($n=5$)	P
T-Cho ($mg\,dl^{-1}$)	21.57±2.05	16.10±0.92	<0.05
TG ($mg\,dl^{-1}$)	57.43±12.13	44.17±4.49	
HDL-C ($mg\,dl^{-1}$)	6.02±1.41	4.89±1.00	

Values represent mean±s.e.mean and statistical significance was assessed by non-paired Student's *t* test.

I/M ratio in rabbits after balloon catheterization

Differences in the I/M ratio are shown in Figure 2. Treatment with BAYw after balloon catheterization resulted in distinct change in the size of intimal thickening. The BAYw group had a smaller I/M ratio and intimal area than the control group (BAYw, 0.29±0.06 ($n=5$); control, 0.65±0.12 ($n=7$); and BAYw, 15.64±3.47 μm^2 ($n=5$); control, 32.01±6.77 μm^2 ($n=7$); respectively, ($P<0.05$). The area of the medial layer was not significantly different between the two groups (data not shown). Figure 3 (a and b) shows a typical EM stain in the control and BAYw groups. The regression of intimal thickening in the BAYw group could be confirmed by the position of the internal elastica lamina.

Immunohistochemical findings in rabbits after balloon catheterization

Immunohistological stainings with SMA revealed diffuse SMA-positive immuno-reactivities throughout in the intimal thickening layers of all preparations (Figure 3 (c and d)). These areas were equivalent to the values of the I/M ratios because of their wide distribution in the neointima. This finding demonstrated that the layers of intimal thickening in this experiment were composed of proliferated and migrated SMCs, and the number of SMCs in those layers was clearly attenuated by the treatment with BAYw.

In addition, immunohistological staining with RAM11 showed scattered immunoreactivity almost exclusively localized in the neointimal layer in the control group (Figure 3 (e)), whereas in the BAYw group the immunoreactivity was markedly less (Figure 3 (f)). Under high magnification, RAM11 immunoreactivity was sporadically detected in the neointimal layer of the control group, but there was very little in the BAYw group (data not shown). The number of RAM11-positive cells in the two groups is shown in Figure 4. In the BAYw group, the number was markedly decreased compared to control (BAYw, 1.05±0.53 ($n=5$); control, 32.46±24.64 ($n=7$), ($P<0.01$).

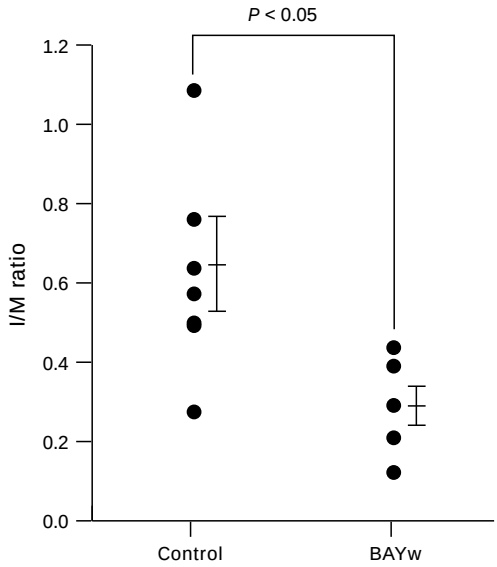

Figure 2 The areas of the intima, measured from the internal elastic lamina to the luminal surface, were calculated by a microscopical image analysing system, XL-10 (OLYMPUS, Tokyo, Japan). The value of the intimal area to medial area was the area ratio of intima/media (I/M ratio) in each group (control group, $n=7$; BAYw group, $n=5$). Results are expressed as mean±s.e.mean, and statistical significance was assessed by Mann-Whitney U test.

DNA content of rabbit carotid artery after balloon catheterization

Total DNA content from the middle piece of the left common carotid artery did not differ between the two groups (control, 0.35±0.03 ($n=7$); BAYw, 0.39±0.02 ($n=5$)).

Discussion

HMG-CoA reductase inhibitors are established agents for the treatment of hypercholesterolaemia (Grundy, 1988; Endo, 1992). Many investigators have shown that HMG-CoA reductase inhibitors have beneficial effects on the regression of atherosclerosis in animals (Kobayashi *et al*., 1989; Zhu *et al*., 1992; Otto *et al*., 1995) and man (Kane *et al*., 1990; Mack *et al*., 1992). However, it was considered that the antiatherosclerotic effect was due to the lipid-lowering properties of these inhibitors. Recently, Soma *et al*. (1993) showed that HMG-CoA reductase inhibitors affect an early event of atherosclerosis *in vivo* and this effect is independent of T-Cho levels.

On the other hand, it is generally recognized that SMC proliferation in the neointima is a major event in the formation of atherosclerosis, including the process of restenosis after successful angioplasty (Ross, 1986; Meier, 1988; Wissler, 1991; Popma *et al*., 1991; Fuster *et al*., 1992). Although the mechanism leading to intimal thickening is complicated and unclear, it is well known that, by some action(s), SMCs in the medial layer might be stimulated and transformed from a contractile type to a synthetic type, and that even they might migrate and proliferate into the intimal layer (Austin *et al*., 1985; Ross, 1986; Wissler, 1991; Lindner & Reidy, 1991; Casscells, 1992). Soma *et al*. (1993) found that carotid arterial myoctyes in the intimal thickening actively incorporated 5-Bromo-2′-deoxyuridine (BrdU), whereas this was not observed in the contralateral sham-operated artery, indicating that proliferated SMCs mainly contribute to the formation of intimal thickening. Their results have led many investigators to attempt to reduce the incidence of restenosis by pharmacological interventions or modified procedures, including the use of

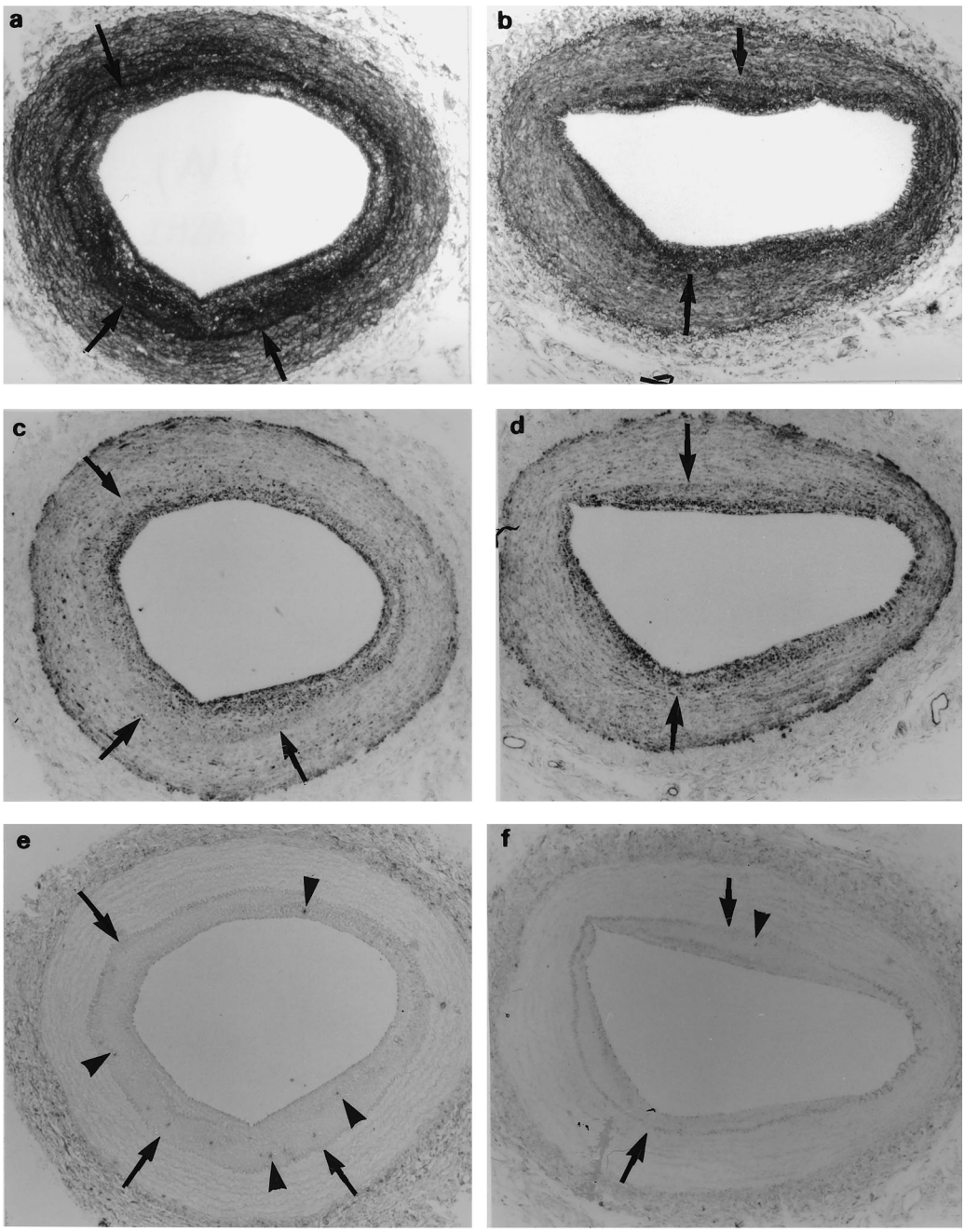

Figure 3 Cross-sections of rabbit left carotid artery after balloon catheterization. They were treated with Elastica Masson (EM) stain, immunohistochemical staining with anti-mouse α-smooth muscle actin antibody (SMA) diluted X2000 with PBS, or anti-rabbit macrophage antibody (RAM11) diluted X50 with PBS by the avidin-biotin-immuno-peroxidase complex (ABC) method; 8% nickel chloride was used as a counterstain. SMA- and RAM11-positive staining shows black. (a) Control; EM stain. (b) Treatment with BAYw6228; EM stain (c) Control; staining with SMA. (d) Treatment with BAYw6228; staining with SMA. (e) Control; staining with RAM11. (f) Treatment with BAYw6228; staining with RAM11. Arrows and arrowheads indicate the position of the internal elastica lamina and RAM11-positive staining, respectively. The preparations were examined under X16 magnification.

angiotensin converting enzyme inhibitors (Jacobsson *et al*., 1994), calcium antagonists (Franklin *et al*., 1993), prostaglandin E_1 derivatives (Motoyama *et al*., 1994) and suramin (Asada *et al*., 1994), and these trials have met with variable success. As for HMG-CoA reductase inhibitors, lovastatin and simbastatin have the effect of reducing the severity of arterial lesions and cardiovascular diseases (Bocan *et al*., 1994). These HMG-CoA reductase inhibitors, not including pravastatin, inhibit SMC proliferation by decreasing the content of mevalonic acid in the cell, and their functions are independent of plasma cholesterol concentrations (Soma *et al*., 1993; Corsini *et al*., 1993). In contrast, Yui *et al*. (1995) showed that pravastatin, if administered before PTCA, may also be useful against highly stenosed or occluded coronary arteries. However, from their studies, it is difficult to extrapolate any direct effect of HMG-CoA reductase inhibitors on SMC growth.

In the present study, we examined the inhibitory effect of BAYw, a newly developed hydrophilic HMG-CoA reductase inhibitor, on the atherogenic process. First, we evaluated the *in vitro* effect of BAYw on the proliferation of SMC from various species, by the measurement of protein content, and demonstrated that the agent markedly inhibited SMC growth in a dose-dependent manner in rats and rabbits (Tables 1 and 2). This effect may be elicited by local inhibition of mevalonic acid synthesis in SMCs. Even in WHHL rabbits, the SMC growth was typically inhibited at lower doses of BAYw than in control

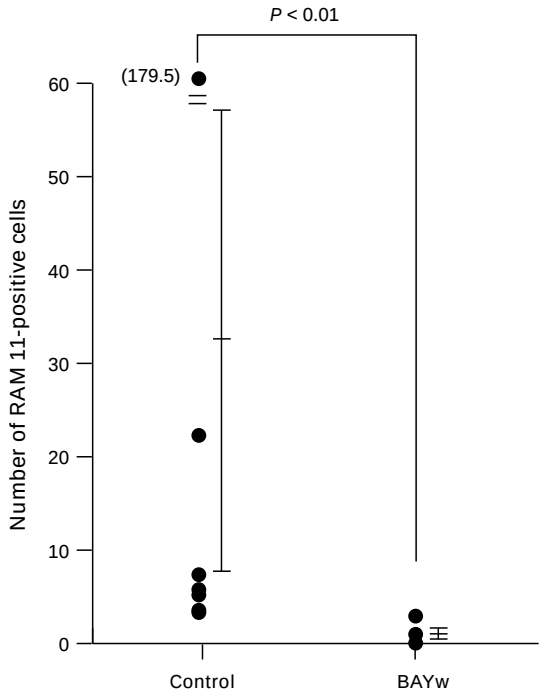

Figure 4 Number of positive cells with anti-rabbit macrophage antibody (RAM11) counted under X100 magnification (control group, $n=7$; BAYw group, $n=5$). Results are

animals (Table 3), suggesting that intimal SMCs from an atheromatous plaque have a high sensitivity to the agent. In human SMC, BAYw also inhibited the growth induced by various stimuli (Tables 4 and 5). It is not clear whether BAYw reduced the SMC proliferation by inhibiting cholesterogenesis or by another effect on SMC. Further studies are necessary to elucidate the mechanism of inhibition by BAYw of SMC proliferation.

Based on these *in vitro* findings, we then examined the *in vivo* effect of the agent to determine whether it could suppress rabbit intimal thickening induced by balloon catheterization. With regard to the effect on T-Cho, although there was a significant difference between the BAYw and control groups, these values were so low that they can be considered insufficient for influencing the formation of intimal thickening. In the *in vivo* study, we observed that neointimal formation induced by the use of two balloon catheterizations to the common carotid artery of rabbits was markedly reduced in the BAYw group as compared with the control group, indicating that the agent has a direct inhibitory effect on carotid intimal thickening (Figures 2 and 3 (a and b)). Furthermore, as the lesions were composed mainly of SMA-positive cells, this suggests that BAYw has a significant inhibitory effect on the content of SMCs in the neointima and the subsequent intimal thickening (Figure 3 (c and d)). These results were obtained with BAYw 1 mg kg^{-1} day^{-1}, a dose much lower than those used in studies with other HMG-CoA reductase inhibitors, such as pravastatin, lovastatin, simvastatin and fluvastatin. Daily doses for rabbits used by Soma *et al.* (1993), were as much as 20 mg kg^{-1}. Differences between the ability of these drugs to penetrate the SMC is a possible mechanism to explain their efficacy of inhibiting SMC proliferation. In addition, the number of macrophages in the neointima with the BAYw group was significantly decreased compared to the control group (Figure 3 (e and f) and Figure 4). Macrophages are known to induce an inflammatory response by producing cytokines such as tumour necrosis factor and interleukin-1 (Tipping & Hancock, 1993), and to induce proliferation and migration of SMCs (Ross, 1986). Although we could not deduce whether the decreased number of macrophages in the neointima was a cause or a result of the administration of BAYw, BAYw might possess the ability to suppress intimal thickening including the regression of SMC content. From these findings, it is reasonable to assume that BAYw prevents progression of atherosclerosis including restenosis after successful PTCA, in contrast to other HMG-CoA reductase inhibitors. In spite of the distinct morphological differences, total DNA content from the intimal thickening was not different between the control and BAYw groups. This discrepancy might be a result of the severe injuries from the two balloon catheterizations.

In conclusion, this study shows that a low dose of BAYw has an effect even on the early events of atherosclerosis and directly inhibits SMC growth, and that this effect is independent of serum cholesterol levels. BAYw, from its properties, may be one of the most promising agents for preventing the progression of atherosclerosis, including complications such as restenosis after PTCA.

References

ASADA, Y., TSUNEYOSHI, A., MARUTSUKA, K. & SUMIYOSHI, A. (1994). Suramin inhibits intimal thickening following intimal injury in the rabbit aorta in vivo. *Cardiovasc. Res.*, **28,** 1166–1169.

AUSTIN, G.E., RATLITT, N.B., HOLLMAN, J., TABEI, S. & PHILLIPS, D.F. (1985). Intimal proliferation of smooth muscle cells as an explanation for recurrent coronary artery stenosis after percutaneous transluminal coronary angioplasty. *J. Am. Coll. Cardiol.*, **6,** 369–375.

BOCAN, T.M.A., MAZUR, M.J., MUELLER, S.B., BROWN, E.Q., SLISKOVIC, D.R., O'BRIEN, P.M., CRESWELL, M.W., LEE, H., UHLENDORF, P.D., ROTH, B.D. & NEWTON, R.S. (1994). Antiatherosclerotic activity of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cholesterol-fed rabbits: a biochemical and morphological evaluation. *Atherosclerosis*, **111,** 127–142.

CASSCELLS, W. (1992). Migration of smooth muscle and endothelial cells. *Circulation*, **86,** 723–729.

CORSINI, A., MAZZOTTI, M., RAITERI, M., SOMA, M.R., GABBIANI, G., FUMAGALLI, R. & PAOLETTI, R. (1993). Relationship between mavalonate pathway and arterial myocyte proliferation: in vitro studies with inhibitors of HMG-CoA reductase. *Atherosclerosis*, **101,** 117–125.

ENDO, A. (1992). The discovery and development of HMG-CoA reductase inhibitors. *J. Lipid. Res.*, **33,** 1569–1582.

FAXON, D.P. & CURRIER, J.W. (1995). Prevention of post-PTCA restenosis. *Ann. New York Acad. Sci.*, **748,** 419–427.

FISCHER-DZOGA, K., JONES, R.M., VESSELINOVITCH, D. & WISSLER, R.W. (1973). Ultra-structural and immunohistochemical studies of primary cultures of aortic medial cells. *Exp. Mol. Pathol.*, **18,** 162–176.

FRANKLIN, S.M. & FAXON, D.P. (1993). Pharmacologic prevention of restenosis after coronary angioplasty: review of randomized clinical trials. *Coronary Artery Dis.*, **4,** 232–242.

FUSTER, V., BADIMON, L., BADIMON, J.J. & CHESEBRO, J.H. (1992). The pathogenesis of coronary artery disease and acute coronary syndrome (1). *N. Eng. J. Med.*, **326,** 242–250.

GRUNDY, S.M. (1988). HMG-CoA reductase inhibitors for the treatment of hypercholesterolemia. *N. Eng. J. Med.*, **319,** 24–33.

HSU, S.M., RAINE, L. & FANGER, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, **29,** 577–580.

JACOBSSON, L.S., PERSSON, K., ABERG, G., ANDERSSON, R.G.G., KARLBERG, B.E. & OLSSON, A.G. (1994). Antiatherosclerotic effects of the angiotensin-converting enzyme inhibitors captopril and fosinopril in hypercholesterolemic minipigs. *J. Cardiovasc. Pharmacol.*, **24,** 670–677.

KANE, P., MALLOY, M.J., PORTS, T.A., PHILLIPS, N.R., DIEHL, J.C. & HARVEL, R.J. (1990). Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens. *J. Am. Med. Assoc.*, **264,** 3007–3012.

KOBAYASHI, M, ISHIDA, F., TAKAHASHI, T., TAGUCHI, K., WATANABE, K., OHNUMA, I & KAMEI, T. (1989). Preventive effect of MK-733 (Simvastatin), an inhibitor of HMG-CoA reductase, on hypercholesterolemia and atherosclerosis induced by cholesterol feeding in rabbits. *Jpn. J. Pharmacol.*, **49,** 125–133.

LINDNER, V. & REIDY, M.A. (1991). Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 3739–3743.

LIU, M.W., ROUBIN, G.S. & KING, S.B. III (1994). Effect of angiographic and hemodynamic results of coronary balloon angioplasty on late angiographic outcome. *Am. Heart J.*, **128,** 1077–1083.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1950). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MACK, W.J., SELZER, R.H., POGODA, J.M., LEE, P.L., SHIRCORE, A.M., AZEN, S.P. & BLANKENHORN, D.H. (1992). Comparison of computer- and human-derived coronary angiographic end-point measures for controlled therapy trials. *Arterioscler. Thromb.*, **12,** 348–356.

MOTOYAMA, Y., SEKI, J., KATOH, Y., NISHIO, M. & YOSHIDA, K. (1994). Effect of TFC-612, a 7-thia prostaglandin E1 derivative, on intimal thickening after endothelial injury with balloon catheter in rats. *Atherosclerosis*, **108,** 159–165.

MEIER, R. (1988). Restenosis after coronary angioplasty: review of the literature. *Eur. Heart J.*, **9,** 1–6.

OTTO, J., ORDOVAS, J.M., SMITH, D., VAN DONGEN, D., NICOLOSI, R.J. & SCHAEFER, E.J. (1995). Lovastatin inhibits diet atherosclerosis in F1B Golden Syrian hamsters. *Atherosclerosis*, **114,** 19–28.

POPMA, J.J., CALIFF, R.M. & TOPOL, E.J. (1991). Clinical trials of restenosis after coronary angioplasty. *Circulation*, **84,** 1426–1436.

ROSS, R. (1986). The pathogenesis of atherosclerosis - An update. *N. Eng. J. Med.*, **314,** 488–500.

SOMA, M.R., DONETTI, E., PAROLINI C., MAZZINI, G., FERRARI, C., FUMAGALLI, R. & PAOLETTI, R. (1993). HMG CoA reductase inhibitors. In vitro effects on carotid intimal thickening in normocholesterolemic rabbits. *Atheroscler. Thromb.*, **13,** 571–578.

TIPPING, P.G. & HANCOCK, W.W. (1993). Production of tumor necrosis factor and interleukin-1 by macrophages from human atheromatous plaques. *Am. J. Pathol.*, **142,** 1721–1728.

WILENTZ, J.R., SANBORN, T.A., HAUDENSCHILD, C.C., VALERI, C.R., RYAN, T.J. & FAXON, D.P. (1991). Platelet accumulation in experimental angioplasty: time course and relation to vascular injury. *Circulation*, **75,** 636–642.

WISSLER, R.W. (1991). Update on pathogenesis of atherosclerosis. *Am. J. Med.*, **91,** 3S–9S.

YUI, Y., KAWAI, C. & HOSODA, S. (1995). Pravastatin (mevalotin) restenosis trial after percutaneous transluminal coronary angioplasty. Cholesterol reduction rate determines the restenosis rate. *Ann. New York Acad. Sci.*, **748,** 208–216.

ZHU, B.Q., SIEVERS, R.E., SUN, Y.P., ISEMBERG, W.M. & PARMLEY, W.W. (1992). Effect of lovastatin on suppression and regression of atherosclerosis in lipid-fed rabbits. *J. Cardiovasc. Pharmacol.*, **19,** 246–255.

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