atherosclerosis.

Although it has been reported that oxidized LDL and LPC activate RhoA in isolated rabbit aortas, which is involved in angiotensin II-induced vasoconstriction, other studies have shown that spontaneously hypertensive rats have higher protein levels of RhoA in tail arteries than control rats[21, 22]. Moreover, vascular Rac1 has been shown to increase in atherogenic animal models *in vivo*[12, 23, 24]. The present study of WHHLMI rabbits clearly demonstrated that the aortic total RhoA and Rac1 levels increased in accordance with the proportions of unprocessed RhoA and Rac1 and the activities of the molecules in the progression of atherosclerosis. This was accompanied by cellular infiltration. Thus, our data showed decreases in total levels, unprocessed levels and the activities of RhoA and Rac1 at 15 and 24 months compared with at 7 months in accordance with the reduction of cellular components[17]. Our *in vitro* study also showed that a significant increase in total RhoA and Rac1 protein levels was induced by oxidized LDL in cultured SMCs, suggesting that hyperlipidemia increases total RhoA and Rac1 protein levels in the vascular wall.

Our previous data showed that the percentages of unprocessed and geranylgeranylated forms of RhoA are approximately 30% and 70% in cultured human aortic endothelial cells, respectively[9]; however, little is known about the proportions of unprocessed and geranylgeranylated forms of RhoA and Rac in the vascular wall *in vivo*. In this study, we found that the proportions of unprocessed and geranylgeranylated forms of RhoA and Rac1 were almost similar to those *in vitro* in early atherosclerotic lesions. Unexpectedly, for the first time we found that the proportions of unprocessed RhoA and Rac1 were increased and reversed by those of geranylgeranylated RhoA and Rac1 in advanced atherosclerotic lesions.

We reported that certain stimulation directly converts almost all unprocessed RhoA into the membrane-bound active form of GTP-RhoA via geranylgeranylation, which exerts vascular responses, including endothelial dysfunction, increased permeability and thrombogenicity[9]. Thus, we expected that hyperlipidemia-dependent atherosclerotic lesions would have greater amounts of geranylgeranylated forms of RhoA and Rac1 than early atherosclerotic lesions; however, the results were the opposite, as described above. Since atherosclerotic lesions with increased cellular components have been characterized by RhoA-dependent endothelial dysfunction and Rac1-dependent ROS generation[1, 11, 12, 23 24], one of the possible implications of our findings is that the increase in the pool of unprocessed forms of RhoA and Rac1 may induce RhoA- and Rac1-dependent vascular responses to atherogenic stimuli. Another important factor may be the time interval of exposure to atherogenic stimuli. The results of *in vitro* experiments in our previous work and the current study were based on short-term exposure to thrombin and oxidized LDL[9]. And long-term exposure experiments might be required to resolve the issue. The detailed mechanisms by which the progression of atherosclerosis exerted increases in unprocessed RhoA and Rac1 remain to be elucidated.

Statins reportedly reduced atherosclerotic lesions with macrophage accumulation in the same animal model used in the present study and directly decreased tissue factor expression in macrophages, independent of lowering cholesterol[25]. We also showed that both hydrophilic and lipophilic statins suppress molecular expression, including tissue factor and plasminogen activator inhibitor-1, by depletion of cellular geranylgeranylpyrophosphate (GGPP)[16, 26-28]. Taken together with other reports[29-31], our previous study clearly demonstrated the exact mechanism of the pleiotropic effect that statins rapidly blocks the activation of unprocessed GDP-RhoA by inhibiting geranylgeranylation[9]. The data from the present study that unprocessed RhoA and Rac1 of advanced atherosclerotic lesions increased may support the potency of the pharmacologial effect of statins, independent of lowering cholesterol. It may be that the more unprocessed RhoA and Rac1 there is, the more effective the statin treatment.

A study limitation was that we showed neither RhoA-dependent nor Rac1/p47^{phox}-dependent vascular responses in this study; however, decreased endothelial cell-dependent arterial relaxation has been demonstrated in WHHL rabbits, and increased superoxide production has been reported in atherosclerotic lesions of WHHL rabbits[32, 33]. Taken together, our results support RhoA-dependent endothelial dysfunction and Rac1-mediated ROS generation in the progression of atherosclerosis in this animal model. Another study limitation was that we could not demonstrate the relation between the increase in unprocessed RhoA and Rac1 in plaques and plaque instability using this novel WHHIMI model; this issue should be extensively investigated. Finally, we could not show the results of membrane translocation at 24 months and GTP-loading at 15 and 24 months due to the limited supply of WHHIMI rabbits since this animal model is valuable.

In conclusion, we provide evidence of marked increases in unprocessed RhoA and Rac1 with increased activity associated with the progression of atherosclerosis in WHHIMI rabbits. This may be the

key to understanding the pathogenesis of hyperlipidemic-dependent atherosclerosis.

Grant Support

This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (14370232, 21590935 and 20790538), Fukushima Medical University Research Project, Takeda Science Foundation, The Uehara Memorial Foundation, Fukuda Foundation for Medical Technology, and a Research Grant for Cardiovascular Diseases from the Ministry of Health, Labor and Welfare of Japan.

References

1) Kaibuchi K, Kuroda S, Amano M: Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem, 1999; 68: 459-486
2) Kamiyama M, Utsunomiya K, Taniguchi K, Yokota T, Kurata H, Tajima N, Kondo K: Contribution of Rho A and Rho kinase to platelet-derived growth factor-BB-induced proliferation of vascular smooth muscle cells. J Atheroscler Thromb, 2003; 10: 117-123
3) Loirand G, Rolli-Derkinderen M, Pacaud P: RhoA and resistance artery remodeling. Am J Physiol Heart Circ Physiol, 2005; 288: H1051-1056
4) van Nieuw Amerongen GP, van Hinsbergh VW: Cytoskeletal effects of rho-like small guanine nucleotide-binding proteins in the vascular system. Arterioscler Thromb Vasc Biol, 2001; 21: 300-311
5) Cai H, Harrison DG: Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res, 2000; 87: 840-844
6) Tzima E: Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. Circ Res, 2006; 98: 176-185
7) Hordijk PL: Regulation of NADPH oxidases: the role of Rac proteins. Circ Res, 2006; 98: 453-462
8) Yokoyama K, Ishibashi T, Ohkawara H, Kimura J, Matsuoka I, Sakamoto T, Nagata K, Sugimoto K, Sakurada S, Maruyama Y: HMG-CoA reductase inhibitors suppress intracellular calcium mobilization and membrane current induced by lysophosphatidylcholine in endothelial cells. Circulation, 2002; 105: 962-967
9) Ohkawara H, Ishibashi T, Sakamoto T, Sugimoto K, Nagata K, Yokoyama K, Sakamoto N, Kamioka M, Matsuoka I, Fukuhara S, Sugimoto N, Takuwa Y, Maruyama Y: Thrombin-induced rapid geranylgeranylation of RhoA as an essential process for RhoA activation in endothelial cells. J Biol Chem, 2005; 280: 10182-10188
10) Rolfe BE, Worth NF, World CJ, Campbell JH, Campbell GR: Rho and vascular disease. Atherosclerosis, 2005; 183: 1-16
11) Ming XF, Barandier C, Viswambharan H, Kwak BR, Mach F, Mazzolai L, Hayoz D, Ruffieux J, Rusconi S, Montani JP, Yang Z: Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction. Circulation, 2004; 110: 3708-3714
12) Laufs U, Wassmann S, Czech T, Münzel T, Eisenhauer M, Böhm M, Nickenig G: Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. Arterioscler Thromb Vasc Biol, 2005; 25: 809-814
13) Shiomi M, Ito T, Yamada S, Kawashima S, Fan J: Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler Thromb Vasc Biol, 2003; 23: 1239-1244
14) Shiomi M, Ito T, Yamada S, Kawashima S, Fan J: Correlation of vulnerable coronary plaques to sudden cardiac events. Lessons from a myocardial infarction-prone animal model (the WHHLMI rabbit). J Atheroscler Thromb, 2004; 11: 184-189
15) Shindo J, Ishibashi T, Yokoyama K, Nakazato K, Ohwada T, Shiomi M, Maruyama Y: Granulocyte-macrophage colony-stimulating factor prevents the progression of atherosclerosis via changes in the cellular and extracellular composition of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits. Circulation, 1999; 99: 2150-2156
16) Ishibashi T, Sakamoto T, Ohkawara H, Nagata K, Sugimoto K, Sakurada S, Sugimoto N, Watanabe A, Yokoyama K, Sakamoto N, Kurabayashi M, Takuwa Y, Maruyama Y: Integral role of RhoA activation in monocyte adhesion-triggered tissue factor expression in endothelial cells. Arterioscler Thromb Vasc Biol, 2003; 23: 681-687
17) Ito T, Tsukada T, Ueda M, Wanibuchi H, Shiomi M: Immunohistochemical and quantitative analysis of cellular and extracellular components of aortic atherosclerosis in WHHL rabbits. J Atheroscler Thromb, 1994; 1: 45-52
18) Okamoto H, Takuwa N, Yokomizo N, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y: Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol Cell Biol, 2000; 20: 9247-9261
19) Yokoyama K, Ishibashi T, Yi-qiang L, Nagayoshi A, Teramoto T, Maruyama Y: Interleukin-1beta and interleukin-6 increase levels of apolipoprotein B mRNA and decrease accumulation of its protein in culture medium of HepG2 cells. J Lipid Res, 1998; 39: 103-113
20) Sakamoto T, Ishibashi T, Sakamoto N, Sugimoto K, Egashira K, Ohkawara H, Nagata K, Yokoyama K, Kamioka M, Ichiki T, Sugimoto N, Kurabayashi M, Suzuki K, Takuwa Y, Maruyama Y: Endogenous NO blockade enhances tissue factor expression via increased Ca^{2+} influx through MCP-1 in endothelial cells by monocyte adhesion. Arterioscler Thromb Vasc Biol, 2005; 25: 2005-2011
21) Galle J, Mameghani A, Bolz SS, Gambaryan S, Görg M, Quaschning T, Raff U, Barth H, Seibold S, Wanner C, Pohl U: Oxidized LDL and its compound lysophosphatidylcholine potentiate AngII-induced vasoconstriction by stimulation of RhoA. J Am Soc Nephrol, 2003; 14: 1471-1479
22) Seasholtz TM, Zhang T, Morissette MR, Howes AL, Yang AH, Brown JH: Increased expression and activity of RhoA

are associated with increased DNA synthesis and reduced $p27^{Kip1}$ expression in the vasculature of hypertensive rats. Circ Res, 2001; 89: 488-495
23) Laufs U, Adam O, Strehlow K, Wassmann S, Konkol C, Laufs K, Schmidt W, Böhm M, Nickenig G: Down-regulation of Rac-1 GTPase by Estrogen. J Biol Chem, 2003; 278: 5956-5962
24) Iwai M, Chen R, Li Z, Shiuchi T, Suzuki J, Ide A, Tsuda M, Okumura M, Min LJ, Mogi M, Horiuchi M: Deletion of angiotensin II type 2 receptor exaggerated atherosclerosis in apolipoprotein E-null mice. Circulation, 2005; 112: 1636-143
25) Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, Shiomi M, Schoen FJ, Libby P: An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation. 2001; 103: 276-283
26) Sakamoto T, Ishibashi T, Sugimoto K, Sakamoto N, Ohkawara H, Niinuma M, Nagata K, Kamioka M, Sugimoto N, Watanabe A, Kurabayashi M, Takuwa Y, Maruyama Y: RhoA-dependent PAI-1 gene expression induced in endothelial cells by monocyte adhesion mediates geranylgeranyl transferase I and Ca^{2+} signaling. Atherosclerosis, 2007; 193: 44-54
27) Ishibashi T, Nagata K, Ohkawara H, Sakamoto T, Yokoyama K, Shindo J, Sugimoto K, Sakurada S, Takuwa Y, Teramoto T, Maruyama Y: Inhibition of Rho/Rho-kinase signaling downregulates plasminogen activator inhibitor-1 synthesis in cultured human monocytes. Biochim Biophys Acta, 2002; 1590: 123-130
28) Nagata K, Ishibashi T, Sakamoto T, Ohkawara H, Shindo J, Yokoyama K, Sugimoto K, Sakurada S, Takuwa Y, Nakamura S, Teramoto T, Maruyama Y: Rho/Rho-kinase is involved in the synthesis of tissue factor in human monocytes. Atherocslesrosis, 2002; 163: 39-47
29) Cordle A, Koenigsknecht-Talboo J, Wilkinson B, Limpert A, Landreth G: Mechanisms of statin-mediated inhibition of small G-protein function. J Biol Chem, 2005; 280: 34202-34209
30) Liao JK, Laufs U: Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol, 2005; 45: 89-118
31) Yoshida M: Potential role of statins in inflammation and atherosclerosis. J Atheroscler Thromb, 2003; 10: 140-144
32) Yamada S, Ito T, Adachi J, Ueno Y, Shiomi M: Decreased arterial responses in WHHL rabbits, an animal model of spontaneous hypercholesterolemia and atherosclerosis. Exp Anim, 2002; 51: 493-499
33) Mollnau H, Schulz E, Daiber A, Baldus S, Oelze M, August M, Wendt M, Walter U, Geiger C, Agrawal R, Kleschyov AL, Meinertz T, Münzel T: Nebivolol prevents vascular NOS III uncoupling in experimental hyperlipidemia and inhibits NADPH oxidase activity in inflammatory cells. Arterioscler Thromb Vasc Biol, 2003; 23: 615-621

Contents lists available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation

Basic research article

Macrophage foam cell formation is augmented in serum from patients with diabetic angiopathy

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ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form
18 October 2009
Accepted 22 October 2009
Published on line 24 November 2009

Keywords:
Macrophage
Diabetic angiopathy
Ex vivo assay
Foam cell

ABSTRACT

The differentiation of macrophages into cytokine-secreting foam cells plays a critical role in the development of diabetic angiopathy. J774.1, a murine macrophage cell line, reportedly differentiates into foam cells when incubated with oxidized LDL, ApoE-rich VLDL or WHHLMI (myocardial infarction-prone Watanabe heritable hyperlipidemic) rabbit serum. In this study, serum samples from Type 2 diabetic patients were added to the medium with J774.1 cells and the degree of foam cell induction was quantified by measuring lipid accumulation. These values were calculated relative to the activities of normal and WHHLMI rabbit sera as 0% and 100%, respectively, and termed the MMI (Macrophage Maturation Index). These MMI values reflected intracellular lipids, including cholesteryl ester assayed by GC/MS. Statistical analysis revealed MMI to correlate positively and independently with serum triglycerides, the state of diabetic retinopathy, nephropathy and obesity, but negatively with administration of α-glucosidase inhibitors or thiazolidinediones. Taken together, our results suggest that this novel assay may be applicable to the identification of patients at risk for rapidly progressive angiopathic disorders.

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Abbreviations: BS, blood sugar; sBP, systolic blood pressure; dBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; SU, sulfonylurea; α-GI, α-glucosidase inhibitor; BG, Biguanide; TZD, thiazolidinedione; eGFR, estimated glomerular filtration ratio; GC, gas chromatography; AUC, area under the curve.

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doi:10.1016/j.diabres.2009.10.011

1. Introduction

Macrophages play important roles in the progression of both diabetic microangiopathy [1,2] and macroangiopathy [3–5]. Macrophages invade the dysfunctional vascular endothelium, change into foam cells by taking up lipids, and secrete high levels of several inflammatory hormones, as well as matrix metalloproteases, which contribute to the development of pathological lesions and remodeling. Indeed, foam cells appear even in the early stages of angiopathy, and the accumulation of large numbers of foam cells is often observed in advanced lesions.

Considering that numbers of patients with diabetic angiopathy and metabolic syndrome are rising dramatically worldwide, it is important to develop a method of identifying those with rapidly progressive angiopathic lesions. In this study, we measured patient serum activity inducing the differentiation of J774.1 into foam cells. During foam cell formation from macrophages, numerous factors including oxidized LDL play an enhancing role [6,7], but J774.1 cells reportedly differentiate into foam cells when incubated with oxidized low density lipoprotein (LDL), very low density lipoprotein (VLDL), or myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbit serum [8]. The WHHLMI rabbit was developed from WHHL rabbits [9], which have a mutated LDL receptor and hypercholesterolemia, such that atherosclerosis develops rapidly [10,11]. The incidence of myocardial infarction in WHHL rabbits was low, while WHHLMI rabbits developed coronary occlusion and spontaneous myocardial infarction reportedly due to having higher amounts of apolipoprotein E-rich VLDL [12–14].

We investigated whether serum activities in diabetic patients inducing the differentiation of J774.1 cells into foam cells, differ according to patient features including metabolic control, development of angiopathic complications, drug treatments, and so on. Our statistical analysis revealed that foam cell-inducing activity correlates positively and independently with serum triglycerides (TG), the state of diabetic retinopathy, nephropathy and obesity, but negatively with administration of α-glucosidase inhibitors (α-GI) or thiazolidinediones (TZD). Such an assay was thus suggested to possibly be applicable to identifying patients at risk for rapidly progressing angiopathic disorders.

2. Materials and methods

2.1. Reagents and cell culture

Murine macrophage-like J774.1 cells were purchased from Riken (Tsukuba, Japan), cultured in RPMI 1640 (Sigma) medium supplemented with 10% fetal calf serum (FCS) (Invitrogen), Penicillin 100 U/ml and Streptomycin 100 μg/ml (GIBCO Invitrogen) at 37 °C in 5% CO_2. All reagents were of analytical grade.

Cells were cultured on 96 well plates (IWAKI) for serum stimulation and lipid accumulation assays. At 90% confluence, each well was incubated with serum free RPMI1640 for 24 h, and then stimulated with 2% serum from individual patients for 72 h. The cells were then subjected to lipid accumulation

Table 1 – (a) Coefficients of correlation and their significance for parametric variables and MMI. (b) Means of MMI for all groups classified by non-parametric complications and use of medications. Values are given as means ± SE. (c) Partial regression coefficients and their significances, for variables adjusted for each other but not normalized. Significance is represented by the *p* values at the bottom.

	Coefficients	*p* value
a.		
Age	−0.025	0.827
BS	0.249	0.030*
HbAlc	0.254	0.027*
sBP	0.068	0.562
dBP	0.075	0.518
WBC	0.246	0.032*
AST	0.012	0.917
ALT	0.134	0.248
γGTP	0.146	0.208
TC	−0.077	0.507
HDL	−0.509	2.55×10^{-6}***
TG	0.528	9.68×10^{-7}***
BMI	0.257	0.025*
Cre	0.259	0.024*
eGFR	−0.188	0.104
	−	+
b.		
Gender (M/F)	19.27 ± 4.97	13.33 ± 1.24
Retinopathy	13.18 ± 1.38	26.07 ± 7.54**
Proteinuria	12.05 ± 1.14	25.46 ± 5.91*
IHD	15.35 ± 1.82	11.55 ± 3.09
SU	14.47 ± 2.57	15.69 ± 2.18
α-GI	15.83 ± 2.03	12.12 ± 2.61
TZD	14.80 ± 1.82	17.88 ± 3.10
BG	12.03 ± 1.25	20.85 ± 4.14*
Insulin	13.10 ± 1.57	18.79 ± 3.87
Anti-RA	11.09 ± 1.11	20.54 ± 3.52*
Statin	13.13 ± 1.27	18.73 ± 4.27
Anti-platelet	14.43 ± 0.96	22.22 ± 15.36
	Coefficients	*p* value
c.		
(Intercept)	19.55	0.544
Age	−0.282	0.029*
BS	0.005	0.869
HbAlc	−1.211	0.388
sBP	0.072	0.286
dBP	−0.129	0.231
WBC	0.043	0.560
AST	0.324	0.140
ALT	−0.196	0.179
γGTP	0.005	0.938
TC	−0.086	0.022*
HDL	0.046	0.593
TG	0.088	3.66×10^{-8}***
BMI	0.590	0.099†
Cre	0.496	0.952
eGFR	0.002	0.986
Gender	0.307	0.926
Retinopathy	9.304	0.029*
Proteinuria	9.363	0.017*
IHD	−7.429	0.152
CVD	3.128	0.728
SU	−1.551	0.541
α-GI	−6.307	0.018*

Table 1 (Continued)

	Coefficients	p value
TZD	−8.273	0.158
BG	4.333	0.088†
Insulin	−1.578	0.637
Anti-RA	−0.641	0.802
Statin	0.789	0.748
Anti-platelet	−4.185	0.312

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
† $p < 0.1$.

assays using AdipoRed (Cambrex), according to the manufacturer's instructions [15]. In brief, the cells were carefully rinsed with phosphate buffered saline (PBS), and 5 μl of AdipoRed reagent were then added to 200 μl of PBS, followed incubation for 10 min at room temperature. Fluorescence was measured with excitation and emission wavelengths of 485 nm and 572 nm, respectively, by fluorimetry. The value obtained with 2% WHHLMI rabbit serum incubation was taken as the Macrophage Maturation Index (MMI) of 100, while 2% normal rabbit serum incubation yielded an MMI of zero. A calibration curve was obtained by serial dilutions of WHHLMI with normal serum. One raw value was the mean, in relative fluorescence units, of five areas per well, and assays were performed five times to obtain a mean MMI value.

2.2. *Serum sampling*

The subjects were 76 patients (53 males and 23 females), who underwent serum sampling in hospitals affiliated with the Institute for Adult Disease, Asahi Life Foundation. The blood examination data and clinical presentations of patients, obtained in routine clinical practice, were collected at the same time as serum sampling. Stage of diabetic retinopathy was determined within 12 months prior to blood sampling, and stage at the last fundus examination was adopted if several examinations had been performed due to stage instability during the prior 12-month period. Patient characteristics are presented in Table 1. All subjects gave informed consent and the study was verified by the institutional ethics committee.

2.3. *Lipid extraction, purification, and separation by GC/MS*

To analyze lipid profiles of accumulated intracellular lipids in J774.1 cells, total lipids were extracted and purified by the Folch method, as previously described [16]. Then, the lipids were dissolved in *n*-hexane and 1 μl of sample was injected into an RTX-5MS (0.25 mm ID × 30 m, 0.25 μm) column attached to a gas chromatograph (Thermo Finnigan Trace GC 2000) which was connected to a mass spectrometer (Thermo Finnigan Trace MS: scanning range: 1–600 *m/z*). The injection temperature was 250 °C, and oven temperatures were 100 °C (2 min) → (25 °C/min) → 250 °C (20 min) → (10 °C/min) → 270 °C (10 min). Cholesteryl components were detected at 362–368 *m/z* independently of other substances.

2.4. *Statistical analysis*

We quantified non-parametrical data such as type of medication, and the state of diabetic complications into indicator variables. Diabetic neuropathy was not assessed because of its variable clinical presentations, which cannot be quantified adequately for transformation into a simple dummy variable and thus could not be effectively utilized in the relatively small number of subjects in this study. Diabetic retinopathy was initially recorded using the Fukuda Classification [17], and stage A2 or later on the most severely affected side, *i.e.* active and progressive states of retinopathy, were dummied as 1, others as 0. Stage of diabetic nephropathy was omitted because diagnosing nephropathy by estimated glomerular filtration ratio (eGFR) and proteinuria was deemed redundant. The presence of ischemic heart disease (IHD) and/or cardiovascular disease (CVD) was determined based on past events, or past and ongoing therapies for these disorders. All ongoing medical therapy classified in Table 1 was dummied as 1 and others 0, dosage-independently.

Data organizing was achieved with Excel2000 (Microsoft), and hierarchical cluster analysis, described using GENESIS 1.7.2 (IGB-TUG). Other statistical analyses were performed using R 2.6.2 (The R Foundation for Statistical Computing), *i.e.* one-way ANOVA, principal component analysis, multi-regression analysis and logistic regression analysis. The Akaike information criterion (AIC) was used to select the best of a collection of candidate models for this dataset.

3. Results

3.1. *Macrophage Maturation Index*

In the macrophage cell line J774.1, administering WHHLMI rabbit serum (final conc. 2%) time-dependently increased macrophage foaming, and lipid accumulation was observed (Fig. 1a) and quantified (Fig. 1b). WHHLMI rabbits exhibit hypercholesterolemia and hypertriglyceridemia, and develop atherosclerosis spontaneously *in vivo* [9]. Their serum thus has the capacity to induce macrophage foaming *ex vivo*. We termed the relative value of lipid accumulation at 72 h, standardized by serial dilution of serum from a WHHLMI rabbit, the MMI. The intra-assay coefficient of variation (CV) was 5.60% ($n = 8$) and the inter-assay CV was 9.43% ($n = 6$). This bioassay was affected by the status of the J774.1 cell line. Thus, assays must be performed with careful attention to cell viability.

We next investigated the MMI of Type 2 diabetic patients whose characteristics are presented in Table 1, and obtained a value of 15.02 ± 14.76 (Supple. 1).

3.2. *Mining the MMI and other datasets*

To clarify the relationships between MMI and other variables, we obtained sera from patients undergoing routine medical investigations. We organized and applied the data to the following analysis. As shown in Fig. 2a, data from all patients were normalized by variables and aligned in ascending order of MMI values. Herein, a tendency for clinical presentation

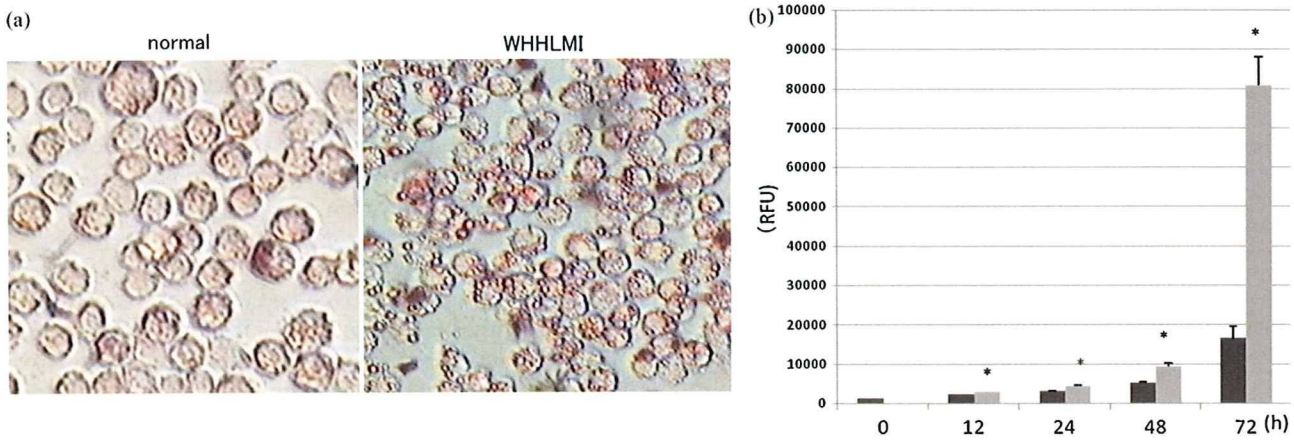

Fig. 1 – (a) Murine macrophage-like cell foaming and lipid accumulation. Cells were cultured on 96-well plates for serum stimulation and lipid accumulation assays. At 90% confluence, each well was incubated with serum free medium for 24 h, and then stimulated with 2% serum from normal and WHHLMI rabbits for 72 h. The cells were then subjected to lipid accumulation assays using Oil red O staining for presentation as an optical microscope image, and using AdipoRed (Cambrex) for quantification. (b) Quantification of lipid accumulation in a time-dependent manner. The cells were carefully rinsed with PBS, and 5 μl of AdipoRed reagent were then added to 200 μl of PBS, followed by incubation for 10 min at room temperature. Fluorescence was measured with excitation and emission wavelengths of 485 nm and 572 nm, respectively, by fluorimetry. One raw value served as the mean of the relative fluorescence, in units, of five areas. The assays were performed five times to obtain a mean MMI value.

with a high MMI to correlate with high Cre, HbA1c, proteinuria and diabetic retinopathy was already apparent, while a low MMI reflected α-GI administration. In Fig. 2b, hierarchical clustering of variables is shown. MMI clustered with TG and renal function. Other variables showed clustering with each other. Only two subjects had CVD, such that power was inadequate for significant variables to be identified by any test. Thus, subjects with CVD were included among those with IHD.

MMI and originally parametric variables are presented in Table 1a. Significant correlations existed between MMI and variables concerning blood glucose control (BS, HbA1c), the inflammatory state (WBC), lipid profile (HDL, TG) and renal function (Cre). When MMI was classified by groups of complications or medications administered, formed retinopathy, proteinuria and administration of BG and anti-RA were associated with significantly higher MMI. Only α-GI administration tended to be associated with lower MMI. With MMI as a criterion variable and others as predictor variables, partial coefficients for regression were calculated, as shown in Table 1c. There was an independent relation between MMI and each variable. Some variables showed inverse correlations or differing significance for the datasets in Table 1a and c, and

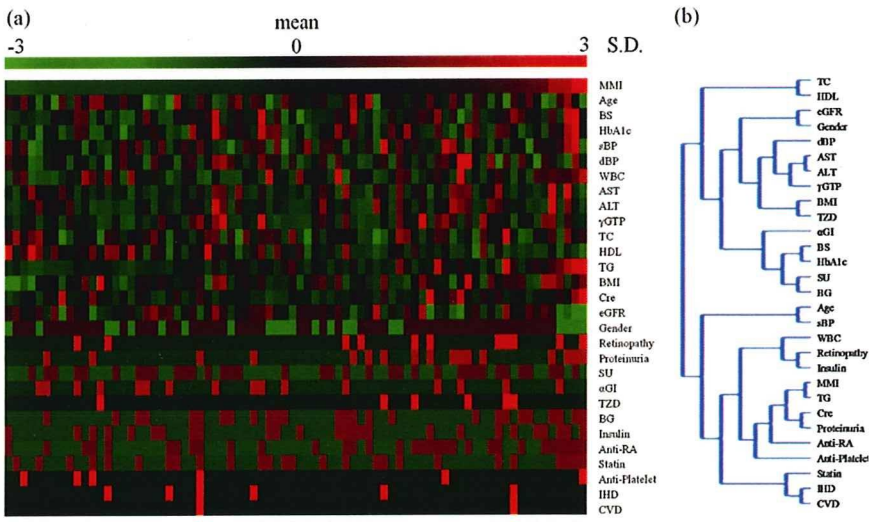

Fig. 2 – (a) Optical presentation of all normalized data from subjects. Parameters were normalized by variables and visualized using GENESIS 1.7.2. Data were aligned in ascending order by MMI from the left for every subject. The tendency was already qualitatively apparent. (b) Hierarchical clustering analysis of variables. Variables presented similar patterns, being clustered in one neighborhood.

Table 2 – Model for multiple regression analysis. Variables were selected by AIC from the variables in Fig. 2. Model: MMI = a × TG (mg/dl) + b × BMI (kg/m^2) + c × (retinopathy) + d × (proteinuria) + e × (α-GI) + f × (TZD) + g.

	Coefficients		Estimated SE	p value
(Intercept)	g	−16.97	5.86	0.0051**
TG	a	0.079	0.0076	8.6×10^{-16}***
BMI	b	0.77	0.24	0.0022**
Retinopathy	c	8.79	2.72	0.0019**
Proteinuria	d	8.28	2.23	0.0004***
α-GI	e	−7.54	2.24	0.0012**
TZD	f	−10.57	3.80	0.0069**

Multiple R^2: 0.75. Adjusted R^2: 0.72.
** $p < 0.01$.
*** $p < 0.001$.

between those in Table 1b and c, probably due to the clinically well-known multi-colinearity (*e.g.* AST and ALT, HDL and TG). In principal component analysis (data not shown), MMI, BS, HbA1c, WBC, HDL, TG, BMI, Cre, retinopathy, proteinuria, BG, Ins, Anti-RA, statin and Anti-Platelet medications were the first principal components.

3.3. *Modeling to account for MMI from routinely obtained parameters*

We subsequently attempted to model a linearly combined collection of variables using stepwise multi-regression analysis. In the model in Table 2, 72% of the variability of MMI was accounted for by variables specified in the model from the adjusted *R*-squared value. Selection of variables was performed using AIC, that is, the relations of MMI to the variables examined in these models were assumed to be stronger than those of residual variables. Values of TG and BMI, the existence of active retinopathy and/or proteinuria, and administrations of α-GI and TZD were determined and their significance was assessed. Coefficients were not standardized in the model, and thus showed gradients for variables reflecting their own measurement units. For instance, a 1 mg/dl rise in TG proportionally indicated an increase of 0.079, diabetic retinopathy an increase of 8.79, and α-GI administration a reduction of 7.54 in MMI. The fitness of the model is described in Supple. 2 as a QQ plot, basically showing a well-fitted and seemingly appropriate model.

TG was the strongest, possibly even excessively influential, variable. However, if our subjects were limited to those with TG values of no more than 300 mg/dl, the TG effect was no longer selected, and BS, AST, HDL and anti-platelet therapy were newly included in the model. BG administration had a reciprocally worsening effect but it was not significant, and diabetic angiopathies and α-GI were still selected, much as in the model shown in Supple. 3. If the strongest TG effect dissipated, 46.51% was still accounted for by the parameters of the adjusted R^2 value despite the smaller subject number.

3.4. *Prediction of diabetic angiopathy activity by MMI*

Sera from patients with both retinopathy and proteinuria exhibited higher MMI than those from patients with either retinopathy or proteinuria (Fig. 3a). To investigate qualitatively whether MMI predicts diabetic angiopathy, a logistic regression analysis was performed (Fig. 3b), using diabetic angiopathy as the criterion variable, and the risk ratio was then estimated. When MMI rose to +1SD (14.76), the risk ratio (RR) for retinopathy was 1.36 (1.10–1.69, 95% C.I.) and the RR adjusted by all other variables was 1.55 (1.06–2.26). The RR for proteinuria was 1.46 (1.18–1.80), the adjusted RR 1.54 (1.10–2.18).

3.5. *Relationships between cholesteryl ester accumulation and MMI*

Relationships of the MMI values and extracted lipid profiles using GC/MS are presented in Supple. 4a and b. In Supple. 4a, a representative GC chart is shown, and the cholesteryl components detected are shown in Supple. 4b. The sum of the AUCs of cholesteryl ester, other lipids (including FFAs, glycerides analyzed from MS data), and total AUCs tended to correlate positively with MMI (Supple. 5).

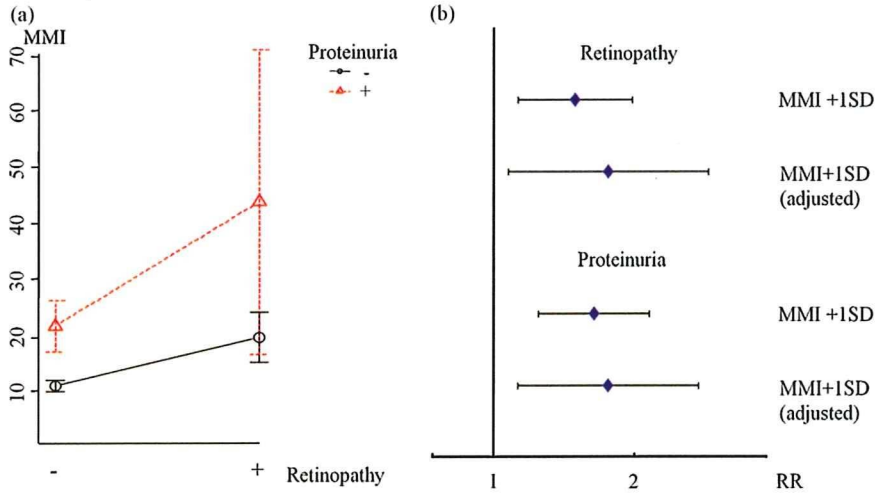

Fig. 3 – (a) Relationships of diabetic angiopathy variables and MMI. Retinopathy and proteinuria together were associated with higher MMI than either retinopathy or proteinuria alone. (b) Risk ratio against increment of MMI (+1SD, 14.76) estimated using logistic regression analysis.

4. Discussion

It is well known that both macroangiopathy and microangiopathy tend to develop rapidly in patients with poorly controlled diabetes. Numerous factors present in macroangiopathy and microangiopathy such as hypertension [18,19], hyperglycemia [20], hyperinsulinemia [21,22], hyperlipidemia [7,23], oxidative stress [24,25] and so on, contribute to angiopathic progression via independent and/or coordinated mechanisms [6,21,24]. Some of these numerous effects act directly on vascular cells [26], while others affect macrophages which invade vascular cells [6]. In this study, we focused on foam cell formation from macrophages, and the activities of patient sera which induce foam cell differentiation were measured. The sera were obtained from diabetic patients, because atherosclerosis and microangiopathy develop rapidly in this patient population. To obtain reproducible values, we used the J774.1 cell line which firmly attaches to culture-dishes and changes into foam cells in response to WHHLMI rabbit serum. Assay reproducibility and validity were confirmed from intra-assay and inter-assay CVs. However, as CVs were still somewhat high, cautious cell handling is critical before proceeding to clinical use. Moreover, by GC analysis, lipid accumulation including cholesteryl ester accumulation rose in proportion to the MMI increase. No specific GC peak corresponding to MMI was detectable. Therefore, the AdipoRed assay precisely reflects intracellular lipid ester accumulation. The obtained values were thus used for comparisons with various clinical factors.

MMI was revealed to be clustered with TG and diabetic nephropathy, the latter being a well-known cause of hypertriglyceridemia [27]. Furthermore, diabetic retinopathy was relatively closely related to MMI and was presumed to be an event preceding nephropathy. Among partial coefficients of regression, age was negative and significant, in fact dovetailing with newly progressive retinopathy being less severe in elderly patients [28].

In multiple regression analysis, we selected six variables which rather strongly accounted for MMI. TG itself is thought to be the primary material of intra-TG accumulation. TZDs might have direct effects against macrophages, while with diabetic retinopathy and nephropathy, administration of an α-GI, which is minimally absorbed from the intestinal tract, and a high BMI reflect only the clinical state and are not directly related to serum parameters. Among these, α-GIs improve blood sugar fluctuations, and a high BMI (obesity) is known to produce metabolic syndrome, the major cause of insulin resistance and atherosclerotic angiopathy. From this viewpoint, it is reasonable that TZDs suppress the ill effects of obesity, by improving insulin resistance. To develop a practical assay, the system shown in the Supple. 3 Table might be worthwhile. It might actually be the case that numerous pathways and bioactive substances affect macrophage maturation in the absence of any lipid effect. Logistic regression analysis, conversely, revealed MMI to be useful for measuring the activity or progression of diabetic angiopathy. In other words, macrophage foam cell formation was related to the development of diabetic microangiopathy. At a minimum, active retinopathy and nephropathy at stage 3a or later were predictable from a higher MMI value at the same time point. Retinopathy and nephropathy acted simply in an additive manner with MMI, suggesting that a higher number of complications reflect the strength of macrophage activity.

Several clinical studies have focused on diabetic angiopathy and medications, with subsets of drugs proving to be effective against angiopathy, though the underlying mechanisms remain uncertain. The α-GIs and TZDs were demonstrated to be effective for the prevention of macrovascular diseases in the stop-NIDDM trial [29] and the PROactive study [30], respectively. However, whether or not this is attributable to direct and/or indirect effects on atherosclerotic regions rather than glycemic control remains unclear. Taking the results of this assay into consideration, these medications may function to prevent macrophage foaming. Moreover, we found that MMI reflected several factors, including foaming or lipid accumulation in individual cells, as well as cell viability and/or growth. Intensive studies focusing on both the characteristics of serum, *i.e.* analysis by 2-DE/mass spectrometry, and the corresponding effects on macrophages, *i.e.* microarray investigations, might clarify the features of macrophage activation.

This study was cross-sectional, such that relationships among variables were ambiguous in terms of cause and effect, especially the impacts of medical therapy. Most of the diabetic patients examined had already received interventional therapies including not only anti-hyperglycemic agents, but also anti-hypertensive agents, statins, anti-coagulants, and so on. Although a future intensive cohort study is needed, MMI might be corrected to avoid excessively strong effects of the lipid state, thus truly reflecting the risk of developing angiopathic diseases in patients with Type 2 diabetes, and thereby become a useful indicator for selecting appropriate medical therapy.

Acknowledgements

We are deeply grateful to Hirokazu Sato and Yuko Oki for their technical supports. This work was supported by a Grant-in-Aid for Exploratory Research from the Japan Promotion of Science Foundation of the Japanese Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.diabres.2009.10.011.

Conflict of interest

There are no conflicts of interest.

REFERENCES

[1] Tesch, Role of macrophages in complications of type 2 diabetes, Clin. Exp. Pharmacol. Physiol. 34 (2007) 1016–1019.
[2] Nguyen, Ping, Mu, Hill, Atkins, Chadban, Macrophage accumulation in human progressive diabetic nephropathy, Nephrology (Carlton) 11 (2006) 226–231.

[3] Boyle, Diabetes mellitus and macrovascular disease: mechanisms and mediators, Am. J. Med. 120 (2007) S12–17.
[4] Simionescu, Implications of early structural-functional changes in the endothelium for vascular disease, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 266–274.
[5] Gacka, Dobosz, Szymaniec, Bednarska-Chabowska, Adamiec, Sadakierska-Chudy, Proinflammatory and atherogenic activity of monocytes in Type 2 diabetes, J. Diabetes Complications (2008).
[6] Hodgkinson, Laxton, Patel, Ye, Advanced glycation end-product of low density lipoprotein activates the toll-like 4 receptor pathway implications for diabetic atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 2275–2281.
[7] Ishigaki, Katagiri, Gao, Yamada, Imai, Uno, et al., Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis, Circulation 118 (2008) 75–83.
[8] Mori, Itabe, Higashi, Fujimoto, Shiomi, Yoshizumi, et al., Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic serum, J. Lipid Res. 42 (2001) 1771–1781.
[9] Shiomi, Ito, Yamada, Kawashima, Fan, Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit), Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1239–1244.
[10] Tanzawa, Shimada, Kuroda, Tsujita, Arai, Watanabe, WHHL-rabbit: a low density lipoprotein receptor-deficient animal model for familial hypercholesterolemia, FEBS Lett. 118 (1980) 81–84.
[11] Buja, Kita, Goldstein, Watanabe, Brown, Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia, Arteriosclerosis 3 (1983) 87–101.
[12] Shiomi, Ito, Shiraishi, Watanabe, Inheritability of atherosclerosis and the role of lipoproteins as risk factors in the development of atherosclerosis in WHHL rabbits: risk factors related to coronary atherosclerosis are different from those related to aortic atherosclerosis, Atherosclerosis 96 (1992) 43–52.
[13] Ishii, Kita, Yokode, Kume, Nagano, Otani, et al., Characterization of very low density lipoprotein from Watanabe heritable hyperlipidemic rabbits, J. Lipid Res. 30 (1989) 1–7.
[14] Ito, Yamada, Shiomi, Progression of coronary atherosclerosis relates to the onset of myocardial infarction in an animal model of spontaneous myocardial infarction (WHHLMI rabbits), Exp. Anim. 53 (2004) 339–346.
[15] Greenspan, Fowler, Spectrofluorometric studies of the lipid probe, nile red, J. Lipid Res. 26 (1985) 781–789.
[16] Kushiyama, Shojima, Ogihara, Inukai, Sakoda, Fujishiro, et al., Resistin-like molecule beta activates MAPKs, suppresses insulin signaling in hepatocytes, and induces diabetes, hyperlipidemia, and fatty liver in transgenic mice on a high fat diet, J. Biol. Chem. 280 (2005) 42016–42025.
[17] Fukuda, Classification and treatment of diabetic retinopathy, Diabetes Res. Clin. Pract. 24 Suppl. (1994) S171–176.
[18] Veglio, Paglieri, Rabbia, Bisbocci, Bergui, Cerrato, Hypertension and cerebrovascular damage, Atherosclerosis (2008).
[19] Silva, Pinto, Biswas, de Faria, de Faria, Hypertension increases retinal inflammation in experimental diabetes: a possible mechanism for aggravation of diabetic retinopathy by hypertension, Curr. Eye Res. 32 (2007) 533–541.
[20] Avogaro, de Kreutzenberg, Fadini, Endothelial dysfunction: causes and consequences in patients with diabetes mellitus, Diabetes Res. Clin. Pract. 82 Suppl. 2 (2008) S94–S101.
[21] Vaidyula, Boden, Rao, Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects, Platelets 17 (2006) 577–585.
[22] Sugimoto, Baba, Suda, Yasujima, Yagihashi, Peripheral neuropathy and microangiopathy in rats with insulinoma: association with chronic hyperinsulinemia, Diabetes Metab. Res. Rev. 19 (2003) 392–400.
[23] Yang, Shi, Hao, Li, Le, Increasing oxidative stress with progressive hyperlipidemia in human: relation between malondialdehyde and atherogenic index, J. Clin. Biochem. Nutr. 43 (2008) 154–158.
[24] Ogihara, Asano, Katagiri, Sakoda, Anai, Shojima, et al., Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase, Diabetologia 47 (2004) 794–805.
[25] Osto, Matter, Kouroedov, Malinski, Bachschmid, Camici, et al., c-Jun N-terminal kinase 2 deficiency protects against hypercholesterolemia-induced endothelial dysfunction and oxidative stress, Circulation 118 (2008) 2073–2080.
[26] Kakehashi, Inoda, Mameuda, Kuroki, Jono, Nagai, et al., Relationship among VEGF, VEGF receptor, AGEs, and macrophages in proliferative diabetic retinopathy, Diabetes Res. Clin. Pract. 79 (2008) 438–445.
[27] Yoshino, Hirano, Nagata, Maeda, Naka, Murata, et al., Hypertriglyceridemia in nephrotic rats is due to a clearance defect of plasma triglyceride: overproduction of triglyceride-rich lipoprotein is not an obligatory factor, J. Lipid Res. 34 (1993) 875–884.
[28] Wong, Molyneaux, Constantino, Twigg, Yue, Timing is everything: age of onset influences long-term retinopathy risk in type 2 diabetes, independent of traditional risk factors, Diabetes Care 31 (2008) 1985–1990.
[29] Chiasson, Josse, Gomis, Hanefeld, Karasik, Laakso, Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial, Lancet 359 (2002) 2072–2077.
[30] PROactive study, Lancet 367 (2006) 982.

Neurourology and Urodynamics

The Effects of Chronic Hyperlipidemia on Bladder Function in Myocardial Infarction-Prone Watanabe Heritable Hyperlipidemic (WHHLMI) Rabbits

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Aims: Lower urinary tract symptoms (LUTS) are common in the aging population. LUTS cause profoundly negative impacts on their quality of life. Pathophysiology of LUTS is multifactorial, and recently, bladder ischemia and metabolic syndrome have been suggested as etiological factors. To evaluate chronic hyperlipidemia on bladder function, we examined the functional and histological changes of the bladder in myocardial infarction-prone Watanabe Heritable Hyperlipidemic (WHHLMI) rabbits. **Methods:** 20- to 24-month-old WHHLMI rabbits and age- and sex-matched control rabbits were prepared. Bladder functions were evaluated using cystometrograms and functional experiments with isolated bladder specimens. Histological studies of bladder and internal iliac arteries were performed with hematoxylin and eosin staining. The bladder was also stained immunohistochemically with mouse monoclonal S-100 antibodies and sheep polyclonal calcitonin gene-related peptide (CGRP) antibodies. **Results:** In cystometric examination, WHHLMI rabbits showed significantly shorter micturition interval, smaller voided volume with non-voiding contractions, and lower micturition pressure, as compared to control. The functional experiments showed that carbachol- and electrical field stimulation-induced contractions were significantly decreased in WHHLMI rabbits than those in control. In WHHLMI rabbits, cross-sections of internal iliac arteries showed significant atherosclerosis and thickening of media. Bladder showed thinner urothelium and decreased smooth muscle area in WHHLMI rabbits, as compared to control. WHHLMI rabbits showed a significant decrease in S-100 protein positive neurons, and an increased number of CGRP positive neurons. **Conclusions:** This study demonstrated that WHHLMI rabbits showed detrusor overactivity with decreased detrusor contraction. It is suggested that chronic hyperlipidemia contributes to the bladder dysfunction. *Neurourol. Urodynam.*

Key words: LUTS; hyperlipidemia; ischemia; WHHLMI rabbits

INTRODUCTION

Lower urinary tract symptoms (LUTS) are common symptoms in the aging population.[1,2] Many people receive profoundly negative impacts on their quality of life from LUTS. The pathophysiology of LUTS is multifactorial, and various etiological factors have been suggested. Recently, metabolic syndrome and bladder ischemia have been suggested as important etiological factors.[3,4] Hyperlipidemia is a well-known risk factor for development and progression for cardiovascular and metabolic diseases. However, association between LUTS and hyperlipidemia is less clear.

Several studies have suggested the changes in bladder function using high cholesterol fed animal with ligation or balloon injury of bladder arteries.[5–7] The reports demonstrated that moderate bladder ischemia caused detrusor overactivity and increase contractile response to carbachol and EFS with moderate fibrosis in the bladder wall, whereas the severe bladder ischemia caused very weak bladder contraction and decreased response to the stimulation.[6] In the experimental model, acute development of atherosclerosis and acute bladder ischemia were induced. Thus, it seems not to be appropriate model for the gradual development of atherosclerosis and ischemia, as it develops in human. Watanabe heritable hyperlipidemic (WHHL) rabbit has been developed as an animal model for human familial hypercholesterolemia and atherosclerosis at first,[8] and now a myocardial infarction-prone Watanabe Heritable Hyperlipidemic (WHHLMI) rabbit as its new strain has been widely used as a model of hyperlipidemia, various organ ischemia, and related diseases.[9–11] Therefore, WHHLMI rabbits might be suitable for evaluation of bladder dysfunction due to chronic hyperlipidemia and slowly progression of atherosclerosis.

In the present study, we examined the functional and histological changes of bladder of WHHLMI rabbits and evaluated the effects of chronic hyperlipidemia and slowly progression of atherosclerosis with bladder ischemia on bladder function.

Conflict of Interest: Yoshida-Consultant: Astellas Pharm., Kissei Pharm., Pfizer. Speaker Honorarium: Astellas Pharm., Kissei Pharm., Pfizer.
Lori Birder led the review process.
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Received 16 August 2009; Accepted 12 October 2009
Published online in Wiley InterScience
(www.interscience.wiley.com)
DOI 10.1002/nau.20843

MATERIALS AND METHODS

Animals

This study was performed according to the Institutional Animal Care and Use in the Ethics Committee of Kumamoto University.

We prepared 20- to 24-month-old WHHLMI rabbits (n = 6), and age- and sex-matched Japanese white rabbits (n = 10) as controls. All WHHLMI rabbits were bred at and donated from the Institute of Experimental Animals of Kobe University School of Medicine. All rabbits were housed individually in metabolic cages in a room temperature and were fed standard rabbit chow (CR-3, Clea, Tokyo, Japan) and water ad libitum for 1 week. For evaluation of blood parameters, blood samples were taken from marginal ear vein after overnight fasting, and were referred to a commercial laboratory (SRL, Tokyo, Japan).

Evaluation of Bladder Function

The urine of each rabbit was automatically captured in a container under the cage, which was connected to an electronic pressure transducer (NA-021, Neuroscience, Tokyo, Japan). The frequency-volume chart (FVC) was recorded and monitored using an electronic pen recorder for 3 days. Then, the bladder was surgically exposed under anesthesia with sodium pentobarbital (35–50 mg/kg) and a catheter (OP-30-05, Eicom, Kyoto, Japan) was inserted into the bladder for cystometric examination, using constant infusion (1.0 ml/min) of saline into the bladder to elicit voiding. The catheter was connected to pressure transducer (TP-400T, Nihon-Kohden, Tokyo, Japan) for measurement of bladder pressure. Saline voided from urethral meatus was collected and measured to determine the voided volume, and the residual volume was measured by aspiration of the residual saline through the intravesical catheter, after the infusion was stopped at the beginning of a voided contraction. In our recording system, we determined bladder contractions over 4 cmH_2O as non-voiding contractions.

Functional Experiments

Under pentobarbital anesthesia, rabbits were sacrificed. Then, bladder was excised, and immersed in Krebs–Henseleit solution. Serosal layer was dissected and bladder strips were cut (approximately 2 mm wide and 8 mm long) from the dome of the bladder. The set up of the bladder specimen was performed as previously described.[12] The bladder strip was suspended in a 20-ml organ bath filled with Krebs–Henseleit solution. Then each preparation was connected to a force displacement transducer (TB-611T; Nihon-Kohden) and an isometric force was recorded and monitored on an ink-writing recorder. Concentration–response curves for carbachol were obtained by increasing the concentration in a stepwise manner after the response to the previous concentration had reached a plateau.

Contractile responses to 80 mM KCl were obtained by equimolar replacement of NaCl by KCl in Krebs–Henseleit solution. Electrical field stimulation (EFS) was generated between two parallel platinum wire electrodes (10 mm wide and 8 mm apart). Electrical impulses for field stimulation of the intramural nervous system of the strips were delivered with a stimulator (SEN-3301; Nihon-Kohden) and boosted by an amplifier (SEG-3104; Nihon-Kohden). The intrinsic nerves were stimulated with rectangular pulses of 0.3 msec duration and 40 V, at stimulation frequencies of 2–40 Hz. Trains of pulses lasted for 2 sec and there was an interval of 2 min between stimulations.

Histological Study

For the histological examination, bladders were fixed by immediate immersion in 0.2 M phosphate-buffered 4% paraformaldehyde (pH 7.4) at 4°C for 4–6 hr. After fixation, they were rinsed and cryoprotected with 10% sucrose in 0.01 M PBS at 4°C for 4 hr to overnight. The fixed specimens were dehydrated in graded ethanol and embedded in paraffin wax. Sections (5 μm) were cut, mounted onto precoated slides, and stained with hematoxylin and eosin (H&E) using standard methods.

To examine pathological changes of bladder vessels, distal portion of internal iliac arteries of two rabbits of both groups were also excised, sectioned transversely, and fixed and mounted onto slides. The prepared slides were stained with H&E.

Formaldehyde-fixed (4%) specimens of bladder in both WHHL (n = 6) and control (n = 10) rabbits were immunohistochemically stained for mouse monoclonal S-100 antibodies (Abcam, Cambridge, UK) and sheep polyclonal calcitonin gene-related peptide (CGRP) antibodies (Biogenesis, England, UK), as previously described.[13,14] During the staining procedure, the sections were pretreated with 3% H_2O_2 for 15 min to remove endogenous peroxidase activity from the tissue and immersed in a solution consisting of 1% bovine serum albumin, 5% NaCl, 1% gelatin, 0.15% glycine, and 1% Tween-20/100 ml of 20 mM Tris–HCl for 5 min to block non-specific background. The treated sections were exposed to the mouse monoclonal antibody S-100 or the sheep polyclonal antibody CGRP at 4°C for overnight. They were then exposed to the goat polyclonal antibody as a secondary antibody for primary antibody (DAKO, Copenhagen, Denmark) for 30 min. The reaction site was stained with diaminobenzidine and counter-stained with hematoxylin for 1 min. The sections were dehydrated through graded alcohols, cleared in xylene, and mounted.

All histological sections were examined using a microscope. A quantitative evaluation of the nerve density in the muscular and stromal layers was calculated, as previously described.[15] In brief, all separate nerve fibers and the constituting nerve fibers in each nerve bundle were counted. In this way, the total number of nerve fibers was obtained. After counting five fields, the mean nerve density score (MNDS) can be calculated.

Statistical Analysis

Values were expressed as mean ± SEM. Statistic analyses for comparisons between groups and between contraction curves were carried out using analysis of variance (ANOVA) and the Fisher's test. $P < 0.05$ was considered as a statistical significance.

RESULTS

There were no significant differences between WHHLMI and control rabbits in body weight, bladder weight, and blood serum examinations except total cholesterol and triglyceride level. Total cholesterol level (496 ± 58 mg/dl, n = 6) and triglyceride level (220.5 ± 22.5 mg/dl, n = 6) in WHHLMI rabbits were significantly higher, as compared to the control (19.5 ± 2.5 and 44.3 ± 4.4 mg/dl, respectively, n = 10). Examination of distribution of cholesterol revealed that WHHLMI rabbits showed three times higher amount of LDL fraction

than that of the control group (60.4 ± 2.8%, n = 6 and 19.2 ± 3.8%, n = 10, respectively).

In FCV, the number of micturition of WHHLMI rabbits (5.9 ± 0.9 times/day, n = 6) was significantly higher than that of the control (2.4 ± 0.6 times/day, n = 10), although the daily urinary volume (WHHLMI rabbits: 75.8 ± 9.3 ml, n = 6; control rabbits: 72.6 ± 10.4 ml, n = 10) was not different between groups. The micturition volume of the WHHLMI rabbits (16.0 ± 5.1 ml, n = 6) was significantly lower than that of the control (43.6 ± 6.9 ml, n = 10).

Typical recordings of the cystometric study in both groups are shown in Figure 1. The micturition volume was significantly lower, and micturition interval was significantly shorter in WHHLMI rabbits. WHHLMI rabbits also showed significantly lower micturition pressure. The post-voided residual urine did not show any significant differences in both groups (Table I).

In the functional study using bladder smooth muscle strips, 80 mM KCl-induced contractions of the control and WHHLMI rabbits (40.2 ± 8.0 mN, n = 10 and 37.6 ± 3.9 mN, n = 6, respectively) were not significantly different. As for carbachol- and EFS-induced contractions, the contractile response was showed as % of KCl-induced contraction. The concentration– and frequency–response curves for smooth muscle strips of WHHLMI rabbits showed substantially weaker contraction to the control (Fig. 2).

In the histological studies, the urothelium of WHHLMI rabbits was thinner than that of the control. Moreover, the amount of muscle fibers decreased and connective tissues increased in the WHHLMI rabbits. In WHHLMI rabbits, the cross-sections of distal portion of internal iliac arteries showed significant atherosclerosis lesions and thickening of media (Fig. 3).

Positive staining for the monoclonal antibody S-100 or the polyclonal antibody CGRP was indicated by red-brown color fibers in bladder of both groups. S-100 positive neurons were mainly detected in smooth muscles layer, and CGRP positive neurons were mainly detected in suburothelium (Fig. 4). WHHLMI rabbits showed significantly lower MNDS of S-100 positive neurons (14.5 ± 3.07, n = 6) and significantly higher MNDS of CGRP positive neurons (30.60 ± 5.43, n = 6), as compared to the control rabbits (29.50 ± 5.22 and 14.9 ± 3.00, n = 10, respectively).

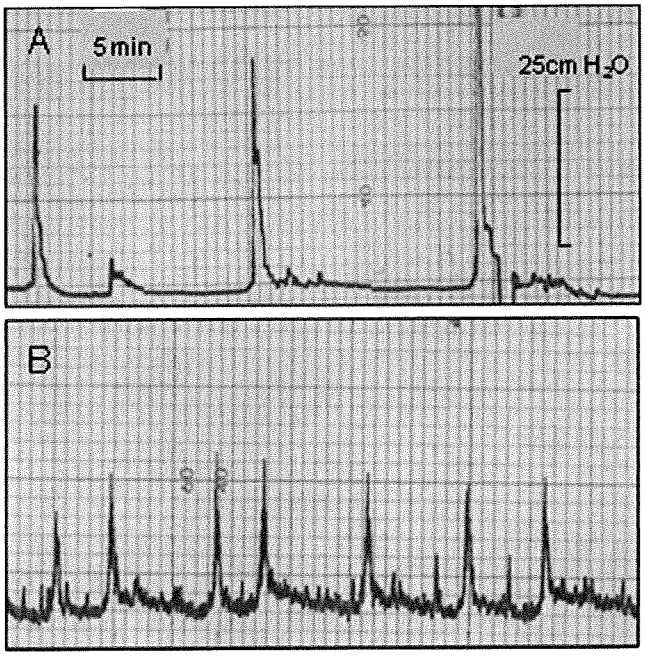

Fig. 1. Typical recordings of cystometrogram. **A**: Control rabbits; **B**: WHHLMI rabbits. WHHLMI rabbits showed shorter interval of micturition and lower micturition pressure, as compared to the control rabbits. There were non-voiding contractions in WHHLMI rabbits.

TABLE I. Comparison of Cystometic Findings Between the WHHLMI rabbits and the Control Group

	WHHLMI (n = 6)	Control (n = 10)
Micturition volume (ml)	6.82 ± 0.87*	20.8 ± 3.2
Interval of micturition (min)	4.52 ± 1.04*	13.87 ± 2.51
Micturition pressure (cmH_2O)	21.8 ± 2.1*	26.8 ± 2.3
Post void residual urine (ml)	1.20 ± 0.41	0.82 ± 0.23

Each value represents mean ± SEM. * $P < 0.05$; significantly different from the comparable value for control.
The bladder was surgically exposed under anesthesia with sodium pentobarbital, and a catheter was inserted into the bladder for cystometric examination, using constant infusion (1.0 ml/min) of saline into the bladder to elicit voiding. The catheter was connected to pressure transducer for measurement of bladder pressure. Saline voided from urethral meatus was collected and measured to determine the voided volume, and the residual volume was measured by aspiration of the residual saline through the intravesical catheter.

DISCUSSION

WHHLMI rabbits were developed as an animal model for myocardial infarction.[9,10] WHHLMI rabbits showed significant atherosclerotic lesions, and aortic atherosclerosis is observed grossly from 4-month old despite being fed normal chow, and the severity of atherosclerotic lesions increased significantly with aging.[9] WHHLMI rabbits are considered to be a good model for research on hyperlipidemia and atherosclerosis, and related ischemic diseases. Additionally, it has been reported that the lipid metabolism and the morphology of the atherosclerotic lesions of WHHLMI rabbits resemble those of humans.[9] Therefore, we used this model in the present experiment. The data of blood biochemistry in this study were consistent with the previous reports.[9–11]

It has been reported that 96% of the WHHLMI rabbits had cerebrovascular atherosclerosis. However, no rabbits showed involvement of penetrating arteries, and stenoses caused by cerebral atherosclerosis generally were milder than those associated with coronary or aortic atherosclerosis. Moreover, no behavioral or morphologic evidence of brain infarction were observed.[11] Hence, it is suggested that bladder dysfunction of WHHLMI rabbits observed in the present study may not be caused by apparent brain disorders. In the present study, we could not evaluate when bladder dysfunction started in WHHLMI rabbits. Evaluation for age-dependent changes of bladder function in WHHLMI rabbits will be needed to clarify it.

In FVC and cystometrogram, WHHLMI rabbits showed frequent voiding and detrusor overactivity (non-voiding contractions). However, micturition pressure of WHHLMI rabbits was significantly lower than that of control. In addition, the functional study with smooth muscle strips from WHHLMI rabbits showed the significantly decreased responses to carbachol and EFS, as compared to control. WHHLMI rabbits showed significant atherosclerosis lesions and thickening of media in the iliac arteries, suggesting slowly progression of ischemia of the bladder. Therefore, in WHHLMI rabbits, the chronic hyperlipidemia and/or atherosclerosis-

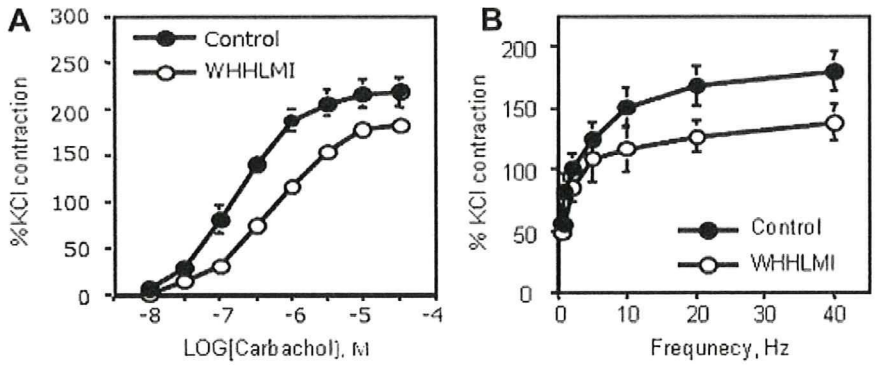

Fig. 2. Concentration–response curves to carbachol (**A**) and frequency–response curves to EFS (**B**) in the isolated detrusor smooth muscles of WHHLMI rabbits (○) and the control rabbits (●). The contractile responses were showed as % of 80 mM KCl-induced contractions. Each point represents the mean ± SEM, if not shown, SEM bars fall within the size of the symbols.

induced poor blood supply to bladder may be related to bladder dysfunction. It is interesting that the WHHLMI rabbits showed almost same results as rabbits with severe bladder ischemia reported by Azadzoi et al.[6] On the other hand, Shenfeld et al.[16] reported that gradual onset of atherosclerosis in apolipoprotein E gene knockout mice did not caused significant changes in bladder smooth muscle contractile responses to bethanechol, KCl, or resting tone. Although differences in the experimental animal and severity of atherosclerosis may contribute to the different results between the report and the present study, further studies will be required to clarify the reasons.

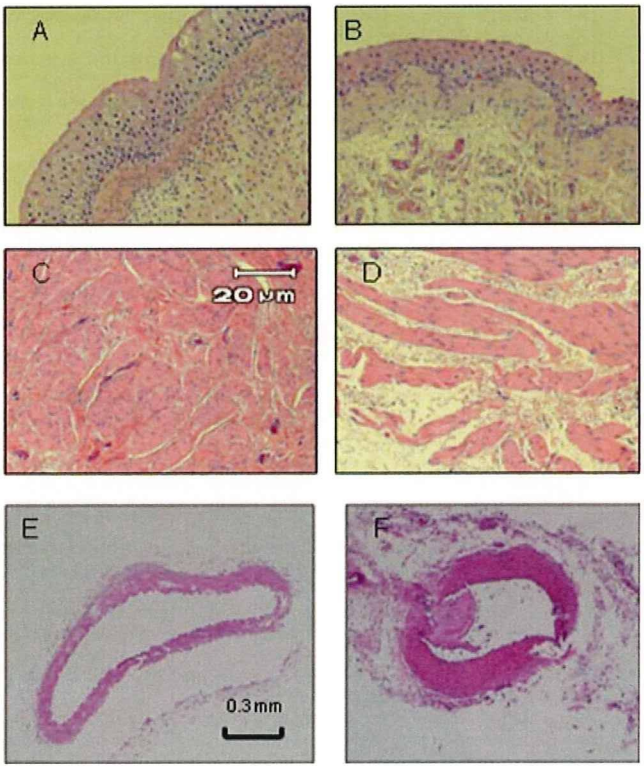

Fig. 3. Histological findings of the bladder and distal portion of internal iliac artery in the control (**A**,**C**,**E**) and WHHLMI (**B**,**D**,**F**) rabbits. The urothelium of WHHLMI rabbits (B) was thinner than that of the control (A), and smooth muscle area of WHHLMI rabbits (D) decreased with replacement by connective tissues. In WHHLMI rabbits, atherosclerosis lesions and thickening of media (F) were observed, as compared to the control (E).

WHHLMI rabbits showed a significant increase in CGRP positive neurons. CGRP is one of the predominant excitatory neurotransmitters in mediating sensory perception, and an important nociceptive marker.[17] CGRP has a major role in mediating hypersensitivity in many systems, including lower urinary tract.[18] The increased CGRP positive neurons in WHHLMI rabbits might cause the enhanced afferent activity, resulting in urinary frequency and detrusor overactivity. Nerve growth factor (NGF) seems to control, at least partly, survival and outgrowth of CGRP positive neurons through its tyrosine kinase receptor A. It has been reported that the increases in NGF and CGRP positive neurons have strong relationship with detrusor overactivity in spinal cord injured rats.[19] The same mechanism may contribute to the detrusor overactivity observed in WHHLMI rabbits. It is suggested that NGF distribution is related to the increase in CGRP positive neurons. Further evaluation will be needed in this point. On the contrary, WHHLMI rabbits showed a significant decrease of S-100 protein positive neurons (denervation), which may contribute to the decreased contractility of bladder smooth

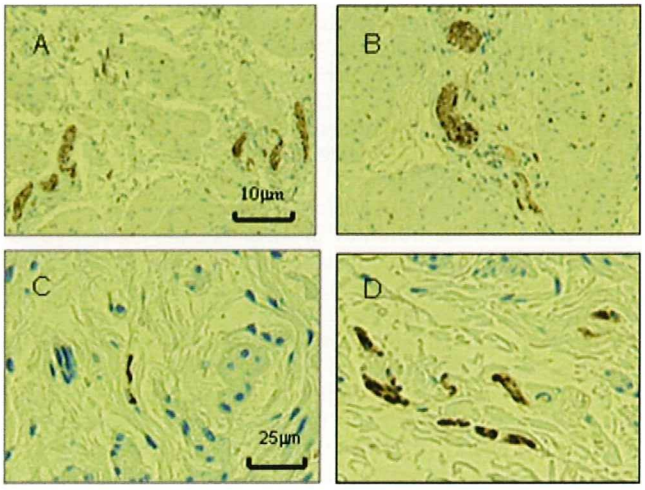

Fig. 4. Immunohistochemical stainings for S-100 positive neurons (**A**: control rabbits and **B**: WHHLMI rabbits) and CGRP positive neurons (**C**: control rabbits and **D**: WHHLMI rabbits). S-100 positive neurons were mainly found in smooth muscle layer, and CGRP-positive neurons mainly found under epithelium. Bladder of the WHHLMI rabbits showed smaller number of S-100 positive neurons (B) and greater number of CGRP positive neurons (D), as compared to the control rabbits (A,C).

muscles to stimulations. Although the mechanism of denervation is not fully understood, Ca^{2+}-dependent neutral protease calpain may be activated by ischemia and result in the proteolysis of neuronal membranes.[20] Histological study of bladder in WHHLMI rabbits showed the fibrosis of bladder wall and the decreased amount of detrusor smooth muscles, which may also contribute to the decreased bladder contractility.

The bladder dysfunction observed in WHHLMI rabbits might be described as the state of detrusor hyperactivity with impaired contraction which can be clinically experienced in human elderly. Although we did not evaluate the time-dependent changes in bladder function in WHHLMI rabbits, bladder dysfunction observed in this study might be a decompensate state after peripheral nervous system activated and reorganized to compensate hyperlipidemia and chronic ischemia with structural an functional changes of bladder. Azadzoi et al. suggested that severe bladder ischemia caused much severe fibrosis, which may be related to a significant increase in the expression of transforming growth factor beta-1 (TGF-β1), and that fibrosis might play a major role in bladder dysfunction.[5] They also speculated that detrusor overactivity observed in rabbits with moderate bladder ischemia was partly because of increased interstitial K^+ concentration in the detrusor, which was derived by decreased K^+ washout due to reduced blood flow.[6] They also suggested that lipoxygenase and cyclooxygenase pathways may affect bladder condition and that leukotrienes may overcome the effect of prostaglandin pathway under ischemic state, resulting in detrusor overactivity.[7]

The unique point in the present study is that bladder weight did not increase and bladder urothelium became thinner; whereas in other experimental models such as BOO, spinal cord injured, and bladder ischemia, bladder weight increased and urothelium appeared thickened, edematous and hyperemic.[5–7,20–22] In addition to the difference in urothelium compensation processes in the various experimental conditions, the presence and degree of inflammation or metabolic changes related to hyperlipidemia may account for urothelium thinning observed in the WHHLMI rabbits, although serum hyperlipidemia alone seems not to cause epithelium thinning.[5,23] Another possibility is the effects of oxidative stress. Reactive oxygen and reactive nitrogen species are known to be generated by ischemia, and they could damage the membrane function.[21] Those changes might promote mucosa thinning and increased permeability of urothelium as well as denervation in bladder wall. Further studies will be needed to clarify this point.

Recent study on WHHLMI rabbits has been shown that the combination of an inhibitor of an acyl-CoA: cholesterol *O*-acyltransferase inhibitor (avasimibe) to a statin (atrovastatin) is able to prevent progression and induce regression of established atherosclerotic lesions.[24,25] Furthermore, it has been reported that control of hyperlipidemia with statins inhibits the progression of kidney disease.[26] Therefore, it might be possible to apply those medicines to retard progression of LUTS or even reduce them. In addition, such study may clarify the mechanism of bladder dysfunction induced by chronic hyperlipidemia and slowly progression of atherosclerosis with bladder ischemia.

CONCLUSIONS

This study demonstrated that WHHLMI rabbits showed detrusor overactivity with decreased detrusor contractility. It is suggested that chronic hyperlipidemia contributes to bladder dysfunction and pathophysiology of LUTS. Furthermore, WHHLMI rabbits may be a useful model for evaluation of pathophysiology of LUTS and exploration of future treatment possibilities.

REFERENCES

1. Homma Y, Yamaguchi O, Hayashi K, The Members of the Neurogenic Bladder Society Committee. An epidemiological survey of overactive bladder symptoms in Japan. BJU Int 2005;96:1314–8.
2. Reeves P, Irwin D, Kelleher C, et al. The current and future burden and cost of overactive bladder in five European countries. Eur Urol 2006;50:1050–7.
3. Rahman NU, Phonsombat S, Bochinski D, et al. An animal model to study lower urinary tract symptoms and erectile dysfunction: The hyperlipidaemic rat. BJU Int 2007;100:658–63.
4. Lee WC, Chien CT, Yu HJ, et al. Bladder dysfunction in rats with metabolic syndrome induced by long-term fructose feeding. J Urol 2008;179:2470–6.
5. Azadoi KM, Tarcan T, Siroky M, et al. Atherosclerosis-induced chronic ischemia causes bladder fibrosis and non-compliance in the rabbit. J Urol 1996;161:1626–35.
6. Azadzoi KM, Tarcan T, Kozlowski R, et al. Overactivity and structural changes in the chronically ischemic bladder. J Urol 1999;162:1768–78.
7. Azadzoi KM, Shinde VM, Tarcan T, et al. Increased leukotriene and prostaglandin release, and overactivity in the chronically ischemic bladder. J Urol 2003;169:1885–91.
8. Watanabe T. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit). Atherosclerosis 1980;36:261–8.
9. Shiomi M, Ito T, Yamada S, et al. Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). Arterioscler Thromb Vasc Biol 2003;23:1239–44.
10. Shiomi M, Fun J. Unstable coronary plaques and cardiac events in myocardial infarction-prone Watanabe heritable hyperlipidemic rabbits: Questions and quandaries. Curr Opin Lipidol 2008;19:631–6.
11. Ito T, Shiomi M. Cerebral atherosclerosis occurs spontaneously in homozygous WHHL rabbits. Atherosclerosis 2001;156:57–66.
12. Yoshida M, Homma Y, Inadome A, et al. Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. Exp Gerontol 2001;36:99–109.
13. Ferrandino I, Grimaldi MC. S-100-immunoreactive nerves in the urinary bladder of the rat. Eur J Histochem 1995;39:127–32.
14. Collins JJ, Wilson K, Fischer-Colbrie R, et al. Distribution and origin of secretoneurin-immunoreactive nerves in the female rat uretus. Neuroscience 2000;95:255–64.
15. Van Poppel H, Stessens R, Baert L, et al. Endoscopic biopsies for quantitative nerve density evaluation of the urinary bladder. Eur Urol 1988;14:236–9.
16. Shenfeld OZ, Meir KS, Yutkin V, et al. Do atherosclerosis and chronic bladder ischemia really play a role in detrusor dysfunction of old age? Urology 2005;65:181–4.
17. Robinson DR, Gebhart GF. Inside information—The unique features of visceral sensation. Mol Interv 2008;8:242–53.
18. Vizzard MA. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. J Chem Neuroanat 2001;21:125–38.
19. Zinck ND, Rafuse VF, Downie JW. Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury. Exp Neurol 2007;204:777–90.
20. Zhao Y, Levin SS, Wein AJ, et al. Correlation of ischemia/reperfusion on partial outlet obstruction-induced spectrin proteolysis by calpain with contractile dysfunction in rabbit bladder. Urology 1997;49:293–300.
21. Juan YS, Lin WY, Kalorin C, et al. The effect of partial bladder outlet obstruction on carbonyl and nitrotyrosine distribution in rabbit bladder. Urology 2007;70:1249–53.
22. Masunaga K, Yoshida M, Inadome A, et al. Prostaglandin E_2 release from isolated bladder strips in rats with spinal cord injury. Int J Urol 2006;13:271–6.
23. Son H, Lee SL, Park WH, et al. New unstable bladder model in hypercholesterolemia rats. Urology 2007;69:186–90.
24. Shiomi M, Ito T, Tsukada T, et al. Combination treatment with troglitazone, an insulin action enhancer, and pravastatin, an inhibitor of HMG-CoA reductase, shows a synergistic effect on atherosclerosis of WHHL rabbits. Atherosclerosis 1999;142:345–53.
25. Worthley SG, Helft G, Corti R, et al. Statin therapy alone and in combination with an acyl-CoA:cholesterol *O*-acyltransferase inhibitor on experimental atherosclerosis. Pathophysiol Haemost Thromb 2007;36:9–17.
26. Fried LF. Effects of HMG-CoA reductase inhibitors (statins) on progression of kidney disease. Kidney Int 2008;100:658–63.

Atherosclerosis 207 (2009) 1–7

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Review

The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: A tribute to the late Dr. Yoshio Watanabe

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ARTICLE INFO

Article history:
Received 8 February 2009
Received in revised form 13 March 2009
Accepted 17 March 2009
Available online 1 April 2009

Keywords:
Atherosclerosis
Hypercholesterolemia
LDL receptor deficiency
WHHL rabbit

ABSTRACT

Professor Yoshio Watanabe, who developed the WHHL rabbit, died on December 13, 2008. He had contributed to studies of lipoprotein metabolism and atherosclerosis, and to the development of hypolipidemic and/or anti-atherosclerotic compounds. WHHL rabbits show hypercholesterolemia due to deficiency of LDL receptors, and very similar lipoprotein metabolism to humans. The incidences of coronary atherosclerosis and myocardial infarction in the original WHHL rabbits were very low. After three rounds of selective breeding, the coronary plaques changed to fibroatheromas with thin fibrous caps and myocardial infarction developed spontaneously. In studies with WHHL rabbits, plaque-stabilizing effects of statins were proved. In this review, we admire his achievements and describe the history of studies using WHHL rabbits.

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1. Introduction

The Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model for hypercholesterolemia due to deficiency of low-density lipoprotein (LDL) receptors and has contributed to studies about lipoprotein metabolism, hypercholesterolemia, and atherosclerosis, and to the development of compounds for treating hypercholesterolemia (especially inhibitors of HMG-CoA reductase, statins) and atherosclerosis. Yoshio Watanabe (Fig. 1), who developed the WHHL rabbit strain, died on December 13, 2008. He was 81 years old.

The first paper about the WHHL rabbit was published in *Atherosclerosis* [1]. After that, many researchers requested WHHL rabbits and Watanabe provided them. One famous study by Goldstein and Brown clarified lipoprotein metabolism in vivo [2]. They proved their LDL receptor pathway hypothesis by using WHHL rabbits and were awarded the Nobel Prize in 1985. In addition, WHHL rabbits have also contributed to the development of statins. As of the end of 2008, a total of 3338 WHHL rabbits have been provided by Kobe University and 603 papers using WHHL rabbits have been published in international journals. A list of these papers appears at the WHHL rabbit-website (http://www.med.kobe-u.ac.jp/iea/w-index.html).

The late Dr. Watanabe was assigned to Kobe University in 1966 to manage a newly constructed animal center. At that time he was 39 years old. As a clinical veterinarian for domestic cattle,

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0021-9150/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2009.03.024

he had been involved in developing a strain of Japanese beef cattle (Tajima cattle) by selective breeding. This experience was to prove useful in developing the WHHL rabbit strain. Although busy establishing a management system for the animal center, Dr Watanabe maintained strong aspirations for research. Seven years later, he accidentally discovered a mutant rabbit showing hypercholesterolemia, from which he developed the WHHL rabbit strain. A man of great patience and perseverance, and strong convictions, he spent almost his entire salary establishing the colony, and devoted himself in maintaining the strain while endeavoring to provide the animals to researchers worldwide. His contribution to the study of lipoprotein metabolism, atherosclerosis, and related diseases is substantial.

In this review, we, as the successors of the WHHL rabbit colony, would like to pay tribute to his scientific achievements by looking back upon the history of studies using WHHL rabbits.

Fig. 1. A photograph of Dr. Yoshio Watanabe taken by Dr. Yoshio Tsujita in 1990.

2. Development of the WHHL rabbit strain

Fig. 2 shows the history of the WHHL rabbit's development. While examining the effects of feeding on serum biochemical parameters in rabbits, Yoshio Watanabe accidentally found a male with hyperlipidemia in 1973. The WHHL rabbit was derived from this mutant. The mutant's serum cholesterol level was 447 mg/dl at 8 months of age despite normal levels of other biochemical parameters except lipids [3]. At that time, there was little interest in hyperlipidemia in Japan. However, Watanabe started to develop a new animal model of the disease. First, he examined whether the hyperlipidemia was heritable and followed Mendel's law. After obtaining the fifth generation of rabbits in 1977, he designated the strain the hyperlipidemic rabbit (HLR) and submitted a paper to a Japanese journal [4]. However, they were not interested. Two years later, he submitted a new

History of the WHHL rabbit's development

1973 Watanabe accidentally finds a mutant rabbit with hyperlipidemia.
(LDL receptor pathway and compactin, the first statin, are found.)
o Watanabe starts breeding using the mutant to develop a novel animal model.

1979 Watanabe establishes the WHHL rabbit strain from the mutant.
o Watanabe starts providing WHHL rabbits to researchers all over the world.
o WHHL rabbits are used in
- studies of lipoprotein metabolism and atherosclerosis
- the development of hypocholesterolemic compounds

1985 WHHL rabbits with a high incidence of coronary atherosclerosis are developed.
o WHHL rabbits are used in studies of
- lipoprotein metabolism, and atherosclerosis
- the antiatherosclerotic effects of statins
- the atherogenicity of oxidative stress

(**In 1990, Professor Watanabe retires** and is succeeded by Dr. Shiomi.)

1992 Coronary atherosclerosis-prone WHHL rabbits are developed.
o WHHL rabbits are used in
- studies of the initiation and progression of atherosclerotic lesions
- studies of the plaque-stabilizing effects of statins
- quantitative analyses of components of atherosclerotic lesions
- the development of hypolipidemic and/or antiatherosclerotic compounds

o Some WHHL rabbits show metabolic syndrome-like features.

1999 Myocardial infarction-prone WHHLMI rabbits are developed.
o WHHLMI rabbits are used in
- the imaging of atherosclerotic lesions
- coronary outward remodeling
- the development of hypolipidemic and/or antiatherosclerotic compounds

Fig. 2. History of the WHHL rabbit's development.

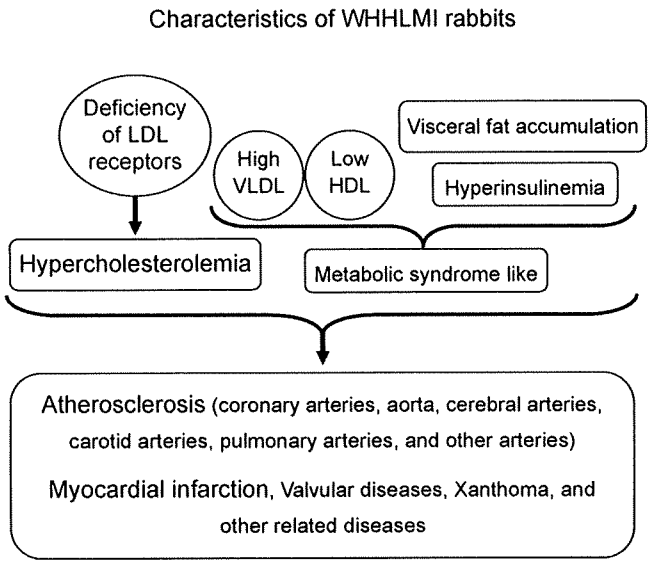

Fig. 3. Characteristics of WHHLMI rabbits.

paper to *Atherosclerosis* which showed the accumulation of β-lipoprotein, aortic atherosclerosis, and xanthoma at the digital joints. In this paper he renamed the strain as WHHL (Watanabe heritable hyperlipidemic) rabbit following a suggestion by Professor Adams C.W.M., one of the chief editors of *Atherosclerosis* [1]. As 1973 was also the year that the LDL receptor pathway was found by Goldstein and Brown [5] and the first statin, compactin [6], was found by researchers of Sankyo Company (Tokyo, Japan), it was an epoch year in the study of lipoprotein metabolism.

3. Lipoprotein metabolism in the WHHL rabbit

Fig. 3 shows the characteristics of the myocardial infarction-prone WHHL (WHHLMI) rabbit, derived from the WHHL rabbit strain. The mechanism of hyperlipidemia in WHHL rabbits was examined from 1980 in collaboration with a research group of Sankyo Company (Japan). Tanzawa et al. [7] found that LDL receptor function was almost deficient in the skin fibroblasts of WHHL rabbits. That study examined the lipoprotein profile of WHHL rabbits and found that almost all of the cholesterol in plasma was accumulated in the LDL fraction. In WHHL rabbits, the disappearance of LDL from plasma was delayed and the LDL-binding activity of skin fibroblasts was almost absent. These results demonstrated that a deficiency of LDL receptor activity resulted in the accumulation of LDL in plasma in this strain. Thereafter, Kita et al. [8] and Attie et al. [9] demonstrated that the LDL receptor activity of WHHL rabbits was deficient in cells of the liver and other major organs. In addition, Goldstein and Brown [5,10–15] and others [16–19] elucidated lipoprotein metabolism in WHHL rabbits. In 1986, Yamamoto et al. [15] demonstrated that 12 nucleotides were deleted in the LDL-binding domain of LDL receptor cDNA in WHHL rabbits. Their study certified that the hypercholesterolemia of WHHL rabbits is due to the genetic defects in LDL receptor and the WHHL rabbit is a true animal model of human familial hypercholesterolemia (FH). In addition, Schneider et al. [14] demonstrated that processing of the LDL receptor from the 120-kDa precursor to the 160-kDa mature form was delayed and many of the mature proteins were destroyed in the cytoplasm.

Compared to mouse models (apoE-KO or LDLR-KO) fed standard chow, WHHL or WHHLMI rabbits resemble humans in lipoprotein metabolism (Table 1). Although plasma cholesterol levels are not very high in mouse models without the feeding of a western diet [20], they are extremely high in WHHL and WHHLMI rabbits (700–1200 mg/dl at 12 months old) similar to human FH. The main lipoprotein in plasma is LDL in WHHL/WHHLMI rabbits and human FH, but it was the VLDL fraction with apoB-48 in apoE-KO mice [20] and HDL and LDL in LDLR-KO mice [21]. The activity levels of cholesterol–ester transfer protein (CETP) in plasma are high in WHHL rabbits [19], although mice and rats do not have the activity [22]. Therefore, HDL levels in plasma are low in WHHL rabbits but high in mice and rats. ApoB-editing enzyme is not expressed in the liver of rabbits [23], although mice and rats do have apoB-editing activity in the liver [20,24]. Therefore, apoB-48-containing very low-density lipoprotein (VLDL) is secreted from the liver in mice and rats. Li et al. [25] demonstrated that apoB-48-containing VLDL particles disappeared from the circulation rapidly supposedly through remnant receptors of the liver similar to chylomicron remnants. As a result, LDL lipid levels in mice and rats are low. In apoE-KO mice, which are hypercholesterolemic, the main lipoprotein fraction was not eluted at the position of the LDL fraction in HPLC and included apoB-48 [26]. Since apoE is a ligand of remnant receptors, apoB-48-containing VLDL particles are not bound to remnant receptors in apoE-KO mice. As a result, apoB-48-containing VLDL accumulates in the plasma of apoE-KO mice. Another hypercholesterolemic model is the LDL receptor-KO mouse. Ishibashi et al. [21] demonstrated that LDL levels were high in LDL receptor-KO mice compared to wild-type mice, and serum cholesterol levels were 225 ± 27 mg/dl in the mice fed standard chow. Such serum cholesterol levels are markedly low compared to levels in human familial hypercholesterolemia homozygotes and WHHLMI rabbits. After the administration of a cholesterol-containing diet, serum lipid levels of LDL receptor-KO mice increased to 1583 ± 120 mg/dl. However, the main lipoprotein fraction was not LDL in HPLC analysis. Therefore, in lipoprotein

Table 1
Differences in lipoprotein metabolism, atherosclerosis, and myocardial infarction among human familial hypercholesterolemia (FH), WHHLMI rabbits, and apoE-KO and LDLR-KO mice fed standard chow.

	WHHLMI rabbits	Human FH	ApoE-KO mice	LDLR-KO mice
Plasma cholesterol levels	Extremely high	Extremely high	Mildly high	Moderate
Main lipoprotein in plasma	LDL	LDL	VLDL	LDL and HDL
LDL levels	Extremely high	Extremely high	Moderate	Moderate
HDL levels	Low	Low	Low	High
ApoB of VLDL	B-100	B-100	B-48	B-48 and B100
Expression of apoB-editing enzyme in liver	No	No	Yes	Yes
Cholesteryl ester transfer activity in plasma	Yes	Yes	No	No
Development of coronary atherosclerosis	Severe	Severe	Resistant	Resistant
Features of coronary atherosclerosis	Various types[a]	Various types[a]	(Not developed)	(Not developed)
Features of aortic atherosclerosis	Complicated lesions	Complicated lesions	Foamy lesions	(Not developed)
Myocardial infarction	Spontaneous	Spontaneous	Resistant	Resistant

[a] Various types consist of lesions showing a large lipid core covered by a thin fibrous cap, fibroatheroma, lesions with intra-plaque hemorrhage and/or calcification, fibromuscular lesions, and foamy lesions.

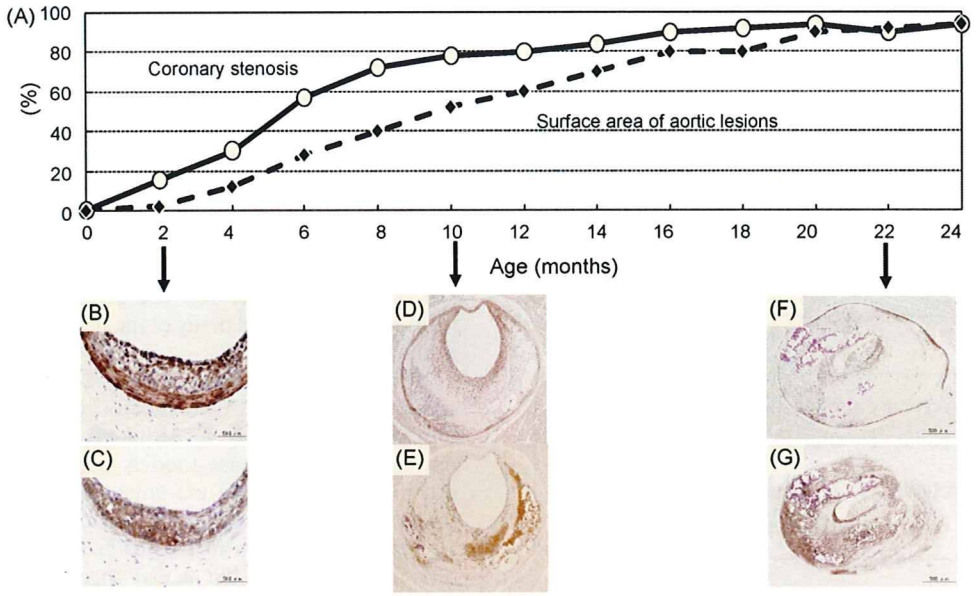

Fig. 4. Progression of atherosclerosis in WHHLMI rabbits. (A) Degree of coronary (cross-sectional narrowing, —) and aortic (percentage of surface damaged in the lumen, - - -) atherosclerosis. (B) 1A4 and (C) RAM-11 immunohistochemical staining of an early coronary lesion. (D) 1A4 and (E) RAM-11 immunohistochemical staining of an established coronary lesion (10 months old). (F) 1A4 and (G) RAM-11 immunohistochemical staining of an advanced coronary lesion (20 months old).

metabolism and the pathophysiological features of hypercholesterolemia, mice are markedly different from humans, but WHHL and WHHLMI rabbits resemble humans.

4. Atherosclerosis

Atherosclerotic lesions develop spontaneously in WHHL and WHHLMI rabbits due to hypercholesterolemia even in animals fed normal chow. In the original WHHL rabbits, prior to 1985, atherosclerotic lesions mainly developed in the aorta and the incidence of coronary atherosclerosis was very low. Therefore, many studies of atherosclerosis were carried out using the aorta of WHHL rabbits. The first detailed analysis was carried out by Buja et al. [27], who showed the accumulation of foam cells derived from macrophages and fibrous caps in the intimal plaques of WHHL rabbits. The mechanism of atherogenesis has been examined histopathologically by using specimens from WHHL rabbits. At the initiation of atherogenesis, arterial endothelial cells express adhesion molecules and circulating monocytes adhere to the arterial endothelial cells [28]. These monocytes infiltrate the sub-endothelial region [27–32] and transform into macrophages. Macrophages express scavenger receptors, remnant receptors and VLDL receptors [33,34] and take in degenerated lipoproteins, such as oxidized lipoproteins, and then transform into foam cells [27–32]. Several research groups [35,36] demonstrated peroxidized lipoproteins in atheromatous lesions of WHHL aortas and that an anti-oxidant, probucol, suppresses the development of atherosclerotic lesions of WHHL aortas. Using ultra-rapid freezing techniques, Frank and Fogelman [37] demonstrated that the diameter of 80% of lipid particles in intimal lesions of WHHL aortas was between 70 and 160 nm. These particles are equivalent to VLDL particles. Recent studies have indicated that remnant lipoproteins including VLDL are atherogenic, similar to oxidized LDL [38]. Foam cells collapse and the accumulated lipids are scattered into the extracellular matrix [39] and then a necrotic lipid core appears [27]. With aging, atherosclerotic lesions grow and the cell components decrease (Fig. 4) [40]. Consequently, atherosclerotic lesions change into rupture-prone plaques having a large lipid core covered with a thin fibrous cap on exposure to risk factors for long periods (Fig. 4F and G), whereas atherosclerotic lesions change into stable fibromuscular lesions on hypolipidemic treatment [41–46]. WHHL rabbits contributed to studies about the initiation and development of atherosclerotic lesions.

5. Coronary atherosclerosis-prone WHHL rabbits

Ultimately an animal model for hypercholesterolemia needs to include myocardial infarction. However, the original WHHL rabbit developed in 1979 did not develop myocardial infarction and had a very low incidence of coronary atherosclerosis [47]. To improve WHHL rabbits as a model for myocardial infarction, Watanabe et al. [47] carried out selective breeding. After five years, the incidence of coronary atherosclerosis was markedly increased. However, the degree of coronary stenosis was mild. Professor Watanabe retired from Kobe University in 1990. His successor attempted to achieve this final goal, the development of myocardial infarction. After a second round of selective breeding, WHHL rabbits with severe coronary atherosclerosis were obtained [48]. However, the incidence of ischemic myocardial lesions was still very low. In a quantitative analysis of the components of atherosclerotic lesions using imaging [40], the coronary plaques of those WHHL rabbits were found to be fibrous and different from the aortic plaques, fibroatheroma. In addition, the cerebral arterial lesions were more fibrous than the coronary lesions in WHHL rabbits [49].

6. Development of myocardial infarction-prone WHHL rabbits

To develop myocardial infarction-prone WHHL rabbits, we selected the descendants of rabbits showing severe coronary lesions mainly consisting of macrophages and foam cells in addition to high plasma cholesterol levels [50] because Van der Wall et al. [51] showed that macrophages and T-lymphocytes accumulated in ruptured plaques in humans. After seven years of selective breeding, we obtained a colony of myocardial infarction-prone WHHL rabbits (designated the WHHLMI rabbit) [50]. The cumulative incidence of myocardial infarction at the age of 30 months was increased from 23 to 97% [50]. Fig. 4 illustrates the progression of atherosclerosis in WHHLMI rabbits [52]. Coronary atherosclerosis is detected from the age of 2 months [52,53]. The lesions are mainly

Table 2
Studies using WHHL or WHHLMI rabbits to develop compounds with hypocholesterolemic and anti-atherosclerotic effects.

	Hypocholesterolemic effects	Anti-atherosclerotic effects	
		Aortic lesion	Coronary lesion
Statin	○	○ ×	○
Anion resin	○	○	n.d.
Statin + resin	○ synergistic	○ synergistic	○ synergistic
Squaren synthase inhibitor	○	○	○
MTP inhibitor	○	n.d.	n.d.
ACAT inhibitor	○ ×	○ ×	○ ×
Probucol	○	○	n.d.
M-CSF and GM-CSF	○	○	n.d.
ApoE	○	○	n.d.
Fibrate	×	n.d.	n.d.
Fish oil or ω3 fatty acids	○ ×	○ ×	n.d.
Thiazolidinedione	×	×	×
Thiazolidinedione + statin	○	○ synergistic	○ synergistic
Ca^{2+} antagonist	×	×	×
β-blocker	×	×	×
ACE inhibitor	×	○	n.d.
A-II receptor antagonist	×	○	n.d.
Gene therapy	○	n.d.	n.d.

○: effective; ×: no effect; ○ ×: inconsistent; n.d.: not determined. References are from the WHHL rabbit-website (http://www.med.kobe-u.ac.jp/iea/w-index.html).

composed of macrophage-derived foam cells. The coronary stenosis (cross-sectional narrowing) was >70% at 10 months and >90% at the age of 20 months [53]. The coronary plaques of WHHLMI rabbits were changed to fibroatheromas from the fibrous lesions of the original WHHL rabbits by selective breeding [50,53]. WHHLMI rabbits showed various coronary plaques [52], including plaques with intra-plaque hemorrhage, calcified nodules, and the denudation of endothelial cells, and fibromuscular lesions. Furthermore, in the coronary plaques, oxidized lipoproteins were accumulated [45,54] and macrophages expressed high levels of matrix metalloproteinases, and interleukin-1 [45,54]. These findings suggest that coronary plaques of WHHLMI rabbits mimic typical human vulnerable plaques. However, no ruptured coronary plaques were detected in WHHLMI rabbits [52]. These observations suggest that not only structural properties of vulnerable plaques but also additional factors or triggers for evoking rupture are required. Myocardial infarction in WHHLMI rabbits is characterized by both new and old infarcts [50].

7. Contribution of WHHL rabbits to the development of compounds for treating hypercholesterolemia and atherosclerosis

It is important that animal models can be used in translational research for human diseases and the development of new drugs, devices, or techniques for therapeutics. WHHL or WHHLMI rabbits have been used in studies of several compounds with hypocholesterolemic and/or anti-atherosclerotic effects (Table 2), including statins, the general term for inhibitors of HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis. More than 20 million patients worldwide take statins, one of the most potent drugs for preventing acute coronary syndromes [55]. Studies using WHHL rabbits elucidated the mechanism whereby a reduction in serum cholesterol levels stabilized atherosclerotic lesions [41–44]. The first statin was compactin [6], found in 1973. Although it was not effective in mice and rats, Watanabe et al. [56] demonstrated dose-dependent hypolipidemic effects of compactin in 1981. After the development of compactin was discontinued, a study using WHHL rabbits [57] showed the hypolipidemic effects of pravastatin, a metabolite of compactin. In 1988, Watanabe et al. [58] demonstrated that lowering serum cholesterol levels with pravastatin suppressed the development of coronary atherosclerosis in WHHL rabbits. Their in vivo study was the first direct evidence of anti-atherosclerotic effects of statins. Furthermore, studies using WHHL rabbits showed that the reduction in serum lipid levels caused by statins altered the composition of coronary plaques from macrophage-rich unstable plaques to fibrous stable plaques [41–44]. In addition, synergistic anti-atherosclerotic effects of treatments combining statins with resin, thiazolidinedione, or ACAT inhibitor, or an angiotensin II receptor inhibitor were also suggested in studies using WHHL rabbits (Table 2). Similar results were obtained in a study of squalene synthase inhibitor, another inhibitor of cholesterol biosynthesis [45].

Studies of the anti-atherosclerotic effects of probucol in WHHL rabbits were dramatic [59,60], demonstrating that oxidative stress plays an important role in atherogenesis and anti-oxidants prevent the development of atherosclerosis. Furthermore, several compounds were examined for hypocholesterolemic or hypolipidemic effects and/or anti-atherosclerotic effects (Table 2). WHHL and/or WHHLMI rabbits have contributed to the development of hypocholesterolemic and/or anti-atherosclerotic drugs. There are two types of studies using WHHL or WHHLMI rabbits to examine the anti-atherosclerotic effects of compounds. One is the plaque prevention study [45,58–60]. The other is the study of plaque-stabilizing effects [41–44]. With the former protocol, treatments are started at 2 months of age when atheromatous plaques are absent or in the early stages, and atherosclerotic lesions are examined at about 10 months when the plaques are established. With the latter protocol, treatments are started from about 10 to 20 months of age when the plaques are unstable and complicated.

Recently, WHHLMI rabbits have been used in studies of the imaging of atherosclerotic lesions by MRI [61], PET [62], and intravascular ultrasound (IVUS) [63]. These techniques are promising for the identification of patients with coronary atherosclerosis and would be useful to prevent acute coronary syndromes.

8. Transgenic WHHL rabbits

Transgenic or knockout mice are useful for studying the functions or roles of genes. Transgenic WHHL rabbits have been developed since 1996 [64]. Genes introduced to date include those for 15-lipoxygenase [64], LCAT [65], lipoprotein(a) [66], lipoprotein lipase [67], and CRP [68]. Interestingly, Fan and Watanabe [69] pointed out that opposite phenotypes were observed for transgenic rabbits and transgenic mice even when the same genes were introduced. Therefore, care is needed when interpreting results from studies with transgenic animals. Transgenic WHHL/WHHLMI