is because of the fact that the Japanese definition requires the central obesity for its diagnosis as in the modified IDF criteria and has more stringent criteria for high fasting glucose for both genders and for HDL cholesterol for women. As shown in this study, the prevalence of MetS in elderly women by the Japanese criteria was very low. This is the reason why we used various analyses using modified ATP III criteria in this cohort. However, the Japanese guideline for MetS was established to identify patients with central obesity, who can reduce the risks by weight loss, whereas the ATP III criteria try to identify patients with multiple risk factors. Therefore, the Japanese criteria should be used to identify obese patients who can have a benefit by weight loss in middle-aged and elderly populations. However, in terms of risk prediction, there have been several reports discussing the cutoff levels of MetS components. Hata et al.[13] have shown significant associations between MetS defined by various criteria and the risk of ischemic stroke in the Hisayama study. In the study, they found that MetS was an independent risk factor for ischemic stroke when they used the modified Japanese criteria with Asian definition of central obesity. Another study from the same group showed that the optimal cutoff level of waist circumference to predict cardiovascular disease was 90 cm in men and 80 cm in women,[19] as we used in modified ATP III definition in this study. Sone et al.[11] also proposed to use the Asian cutoff for waist circumference to define central obesity from the data of Japan Diabetes Complication Study. In terms of the appropriate cutoff level of HDL cholesterol for the definition of MetS in Japanese women, not so many analyses have been done. In our study, the prevalence of low HDL cholesterolemia with the cutoff of 40 mg/dL was less than 5% and was approximately 20% with the cutoff of 50 mg/dL in elderly women. We previously showed that central obesity was significantly associated with low HDL cholesterolemia only when we used the cutoff of 50 mg/dL for women.[5] Therefore, further study is necessary to determine the appropriate cutoff level of HDL cholesterol in women.

In summary, we have shown the prevalence of MetS in Japanese elderly and middle-aged population using Japanese and modified ATP III and IDF criteria, and found the effect of aging on the prevalence only in women with either criterion. We also showed the effect of aging on each metabolic component in this cohort. Thus, aging is an important factor that affects the metabolic abnormality, and aging of the population would lead to the increase in the prevalence of MetS. Therefore, the development of better approaches to the prevention and management of MetS is necessary for successful aging in our society.

Acknowledgments

This study was supported by research grants for health sciences from the Japanese Ministry of Health, Labour and Welfare and by Osaka Pharmaceutical Manufacturers Association. The authors also thank Dr Hiroshi Kanamori (Hyogo Prefectural Amagasaki Hospital) for his help in the statistical analysis.

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Bioorganic & Medicinal Chemistry 18 (2010) 3946–3950

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Liver-targeted siRNA delivery by polyethylenimine (PEI)-pullulan carrier

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ARTICLE INFO

Article history:
Received 5 March 2010
Revised 12 April 2010
Accepted 13 April 2010
Available online 18 April 2010

Keywords:
Polyethylenimine
Gene delivery
Cationic polymer
Cytotoxicity

ABSTRACT

Recently, small interfering RNA (siRNA)-based therapeutics have been used to treat diseases. Efficient and stable siRNA delivery into disease cells is important in the use of this agent for treatment. In the present study, pullulan was introduced into polyethylenimine (PEI) for liver targeting. PEI/siRNA or pullulan-containing PEI/siRNA complexes were delivered into mice through the tail vein either by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with an increase in the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. Moreover, the hydrodynamics-based injection increased mice mortality. Introduction of pullulan into PEI dramatically reduced mouse death after systemic injection. After systemic injection, the PEI/fluorescein-labeled siRNA complex increased the level of fluorescence in the lung and the PEI-pullulan/siRNA complex led to an increased fluorescence level in the liver. These results suggest that the PEI-pullulan polymer may be a useful, low toxic means for efficient delivery of siRNA into the liver.

1. Introduction

Small interfering RNA (siRNA)-based therapeutics, which are now recognized as a medical approach for the treatment of difficult-to-cure diseases such as viral infections and tumors, are attracting considerable attention in recent times.[1,2] However, naked siRNA is unstable in the bloodstream and is rapidly eliminated through the urinary system. Moreover, its negative charge inhibits efficient cellular uptake due to the negative charge of the cell surface. Thus, efficient and stable siRNA delivery into diseased cells is critical in this treatment modality. Many researchers have attempted to induce various chemical modifications into siRNA or to form complexes with several cationic carriers such as cationic polymers, liposomes, peptides, or proteins.[3–5]

Among cationic polymers, polyethylenimine (PEI) is the most popular synthetic polymer and has a high cationic charge density. It has been widely used to deliver siRNAs into cell lines or tissues. Naked siRNAs are unstable and are rapidly degraded, but PEI is able to form stable complexes with siRNAs, leading to the protection of genes from enzymatic degradation. Moreover, PEI shows a strong buffer capacity over a wide range of pH values; this plays an important role in the escape of genes from the endosome after endocytosis. On the other hand, the high cationic density of PEI allows for the formation of highly condensed complex with siRNAs, but complex formation with PEI can lead to cytotoxicity.[6–10] Information on the safety and biodistribution of PEI or PEI/siRNA complexes both in vitro and in vivo would contribute to improving the safety and efficiency of siRNA delivery using PEI.

In the present study, we introduced pullulan into PEI. Pullulan is a water-soluble polysaccharide consisting of three α-1,4-linked glucose polymers with different α-1,6-glucosidic linkages. It is used for liver targeting because of its high affinity for the asialoglycoprotein receptor in the liver.[11–13] We delivered PEI/siRNA or pullulan-containing PEI/siRNA complexes into mice through the tail vein by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with increasing the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. On the other hand, the introduction of pullulan into PEI reduced mouse mortality and increased liver-targeting efficiency.

2. Results and discussion

2.1. Polymers

A linear 22-kDa PEI was used for the synthesis of the siRNA and PEI-pullulan polymer complex (Fig. 1). The amount of pullulan in

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doi:10.1016/j.bmc.2010.04.031

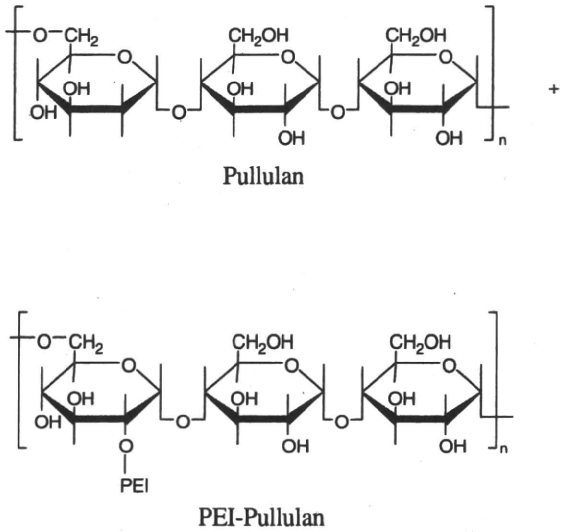

Figure 1. Chemical structure of pullulan and PEI-pullulan. To synthesize the PEI-pullulan polymer, 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) were stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature and then 13.2 mg of linear PEI (M_w, 22 kDa; 0.3 mmol) was added to the mixture.

the polymer was estimated to be 39 mol % and molecular weight of polymer was 2.6×10^5 (see Supplementary data). The zeta potentials of polymer/siRNA complex increased with increasing N/P ratio and showed nearly neutral at N/P ratios of 48 and 96 (see Supplementary data).

2.2. Measurements of complex diameters

The complexes of polymer and siRNA were prepared at several N/P ratios (1.5, 3, 6, 12, 24, and 48) and were determined using a Zetasizer. The particle size decreased with increasing N/P ratio. PEI/siRNA complexes showed <200 nm for all N/P ratios, whereas PEI-pullulan/siRNA complexes with ratios of 12 to 48 were <200 nm (Fig. 2).

2.3. Electrophoresis of the polymer/siRNA complex

Polymers were mixed with siRNA at several N/P ratios. The complexes were analyzed by electrophoresis. Bands corresponding

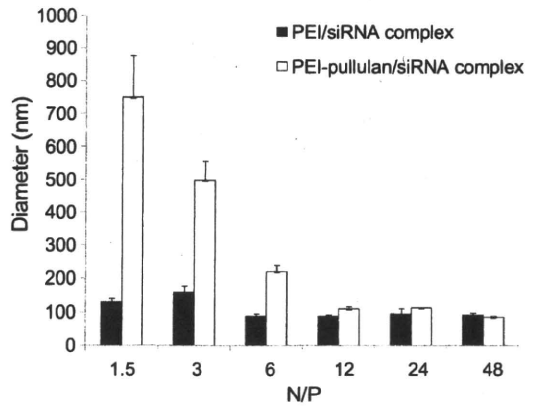

Figure 2. Diameter of the PEI/siRNA or PEI-pullulan/siRNA complexes. Polymer and siRNA complexes were simply prepared by incubating siRNA and polymer in water. The diameters of the complexes were determined using a Zetasizer.

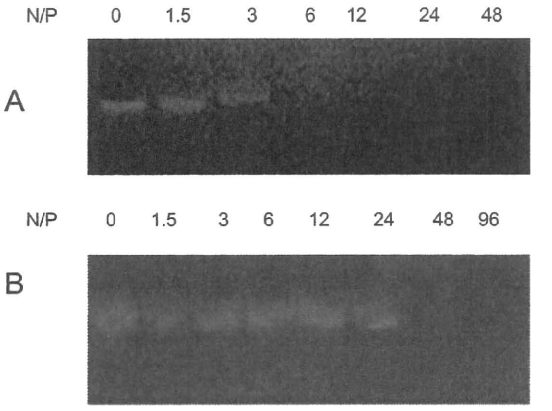

Figure 3. Electrophoresis of (A) PEI/siRNA and (B) PEI-pullulan/siRNA complexes. Various concentrations of the polymer were mixed with the siRNA and analyzed by 19% polyacrylamide gel electrophoresis. A N/P ratio of 0 implies siRNA alone.

to free siRNA in the PEI/siRNA complex were not observed when the polymer was present at N/P ratios of above 3, whereas when the N/P ratios were 1.5 and 3, bands corresponding to free siRNA were observed. In the case of the PEI-pullulan/siRNA complex, no suppression of siRNA was indentified in those complexes with N/P ratios of 1.5 to 24, while siRNA migration in complexes with N/P ratios of $\geqslant$48 was suppressed (Fig. 3). These results show that introduction of pullulan into PEI weakens the polymer and siRNA complex.

2.4. Safety of polymer/siRNA complexes in vivo

PEI alone, the PEI/siRNA complex, and the PEI-pullulan/siRNA complex were injected into mice using a hydrodynamics-based or a non-hydrodynamics-based procedure. PEI alone or the PEI/siRNA complex with high N/P ratios ($\geqslant$6.0) increased mice mortality after systemic injection using the non-hydrodynamics-based procedure (Fig. 4); note that all mice died when complexes with N/P ratios of $\geqslant$12 were injected (data not shown). Similarly, previous studies reported that the PEI/DNA complex with a N/P ratio of 6 resulted in the death of 50% of the injected mice.[14,15] However, all mice died when PEI alone or the PEI/siRNA complex with a N/P ratio of 3 was injected using the hydrodynamics-based procedure. Hydrodynamics-based transfection was developed to deliver naked DNA or RNA into the liver by intravenous injection of a large volume of DNA or RNA solution at high velocity. This is an efficient method for liver-specific in vivo gene delivery.[16,17] However, in our study, high mouse mortality was observed when the hydrodynamics-based procedure was used for the in vivo delivery of PEI/siRNA complexes.

All dead mice lapsed into dyspnea less than 30 min after injection and showed hemorrhage-like dark red regions in the lung. There was no difference in mortality between mice injected with PEI alone and those injected with the PEI/siRNA complex, but more severe hemorrhage-like dark red regions were observed in the former (Fig. 4A and B).

Concerning the death of mice after systemic injection, Fahrmeir's group suggested that free PEIs after complex formation with DNA correlate with mouse mortality.[18] Several studies showed that increased gene expression in the lung is associated with lung damage and mouse mortality after intravenous injection of PEI/DNA or modified PEI/DNA.[15,19,20] In the present study, PEI/siRNA showed a similar in vivo toxicity to PEI/DNA.

On the other hand, no mortality was observed in mice injected with PEI-pullulan/siRNA complexes with N/P ratios of 6 to 48 by the hydrodynamics-based procedure mice (Fig. 4B) and the non-hydrodynamics-based procedure (data not shown). These

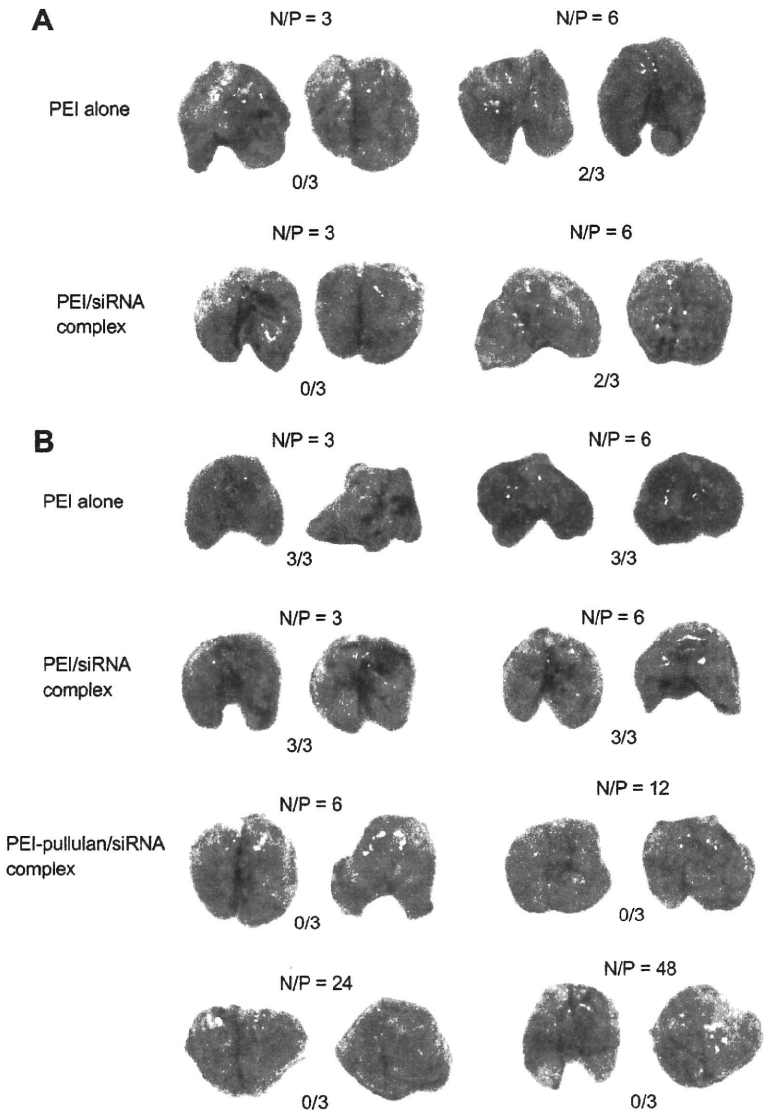

Figure 4. Delivery of PEI alone or polymer/siRNA complexes into mice by using the (A) non-hydrodynamics- or (B) hydrodynamics-based procedure. Numbers of dead mice per total mice are described below.

results suggest that intravenous injection with PEI alone or the PEI/siRNA complex at high N/P ratios can increase mortality, but introduction of pullulan into PEI results in low mortality. Moreover, hydrodynamics-based injection can increase the mouse mortality rate, compared to non-hydrodynamics-based injection. High in vivo toxicity or mortality caused by systemic injection of the PEI-based complex is an obstacle to be overcome. Many research efforts such as the introduction of poly(ethylene glycol) (PEG)[15] and removal of free PEIs after complex formation[18] were reported to efficiently reduce in vivo toxicity or mortality. In the present study, introduction of pullulan to PEI dramatically reduced in vivo toxicity and mortality.

2.5. Biodistribution after injection of the polymer/siRNA complex into mice

siRNA formed a complex with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. Complexes were injected into the mice via the tail vein using the non-hydrodynamics-based procedure. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected at 1 or 3 h after the injection. At 1 h after the injection of the PEI/siRNA or PEI-pullulan/siRNA complex, fluorescence was identified mainly in the lung and kidney. At 3 h, fluorescence increased in the livers of the PEI-pullulan/siRNA complex-injected mice, but was barely found in the livers of the PEI/siRNA-injected mice (Fig. 5).

Several studies have reported that linear and branched PEI/gene complexes show different biodistribution and transfection efficiency.[6–9] The linear PEI/gene complex exhibits more efficient transgene expression in the lung when injected intravenously, as compared to the branched PEI/gene complex;[6,7,9,14,21] however the transgene expression of the branched PEI/gene complex may be more efficient in other tissues (e.g., kidney).[9,22] Further, although PEI cytotoxicity depends on molecular weight and N/P ratios, the branched PEI/gene complex is found to have higher toxicity or cause more tissue damage as compared to the linear PEI/gene complex.[8,9,23]

In the present study, we used a linear 22-kDa PEI for complex formation with siRNA and for synthesizing the PEI-pullulan

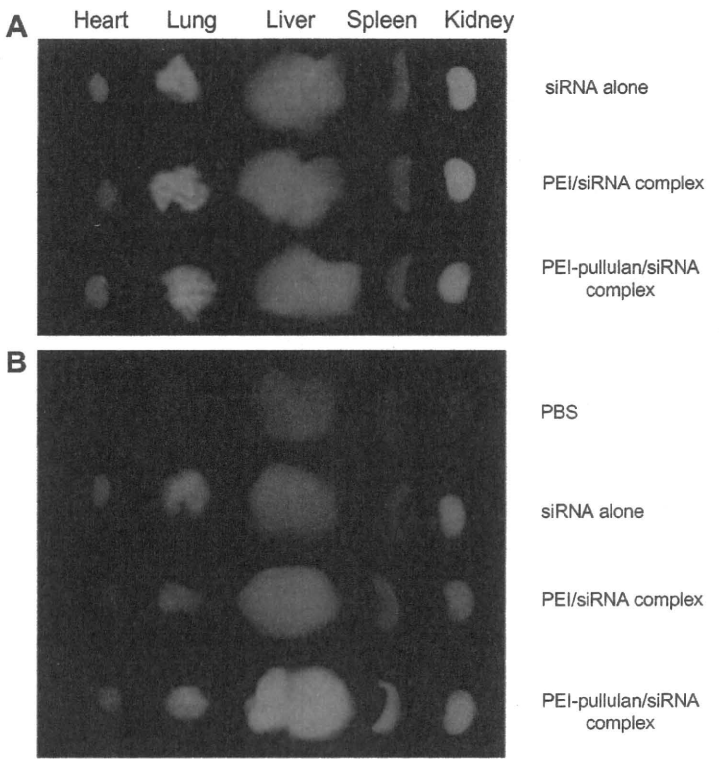

Figure 5. Biodistribution after injection of PBS, siRNA alone, or polymer/fluorescein-labeled siRNA complexes. The siRNA was bound with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected (A) 1 or (B) 3 h after the injection.

polymer. When the 22-kDa linear PEI/gene complexes were transfected via systemic administration, the main target was the lung and lower levels of transfection were found in the brain, heart, liver, spleen, and kidney.[14] High transgene expression in the lungs may relate to rapid crossing of the pulmonary endothelial barrier by the PEI/gene complexes.[21] Similarly, we found the highest level of fluorescence in the lung compared to other tissues (heart, liver, and spleen) at 1 h after intravenous injection of the PEI/siRNA complex at a N/P ratio of 3 (Fig. 5). Fluorescence in the kidney may be caused by elimination of biodegraded free fluorescein from the system.

siRNA-based therapeutics are recognized as a useful approach for liver (hepatic) diseases such as hepatitis B and C, but development of liver-targeted siRNA delivery system is an important problem to solve.[1] In the present study, pullulan, a water-soluble polysaccharide, was introduced into PEI to increase liver-targeting efficiency. At 3 h after the injection, we found highest level of fluorescence in the livers of the PEI-pullulan/siRNA complex-injected mice (Fig. 5). Thus, our system may be a useful means for efficient delivery of siRNA into the liver.

3. Conclusions

We found that introduction of pullulan to PEI increased the level of fluorescence in the liver. This finding may be explained by the fact that pullulan has a high affinity for asialoglycoprotein receptors in the liver.[11–13] Moreover, systemic delivery of PEI-pullulan polymer dramatically reduced mouse death. These results suggest that the PEI-pullulan polymer may be an efficient and low toxic means for siRNA delivery into the liver.

4. Materials and methods

4.1. Fluorescein-labeled siRNA

The gene (*apoB* siRNA) used in this study was amidated and its sequence was as follows: 5′-GUCAUCACACUGAAUACCAAUdTdT-3′ (sense) and 5′-dTdTCACAGUAGUGUGACUUAUGGUUA-3′ (antisense). Alexa Fluor 750 (Invitrogen, Tokyo, Japan) was used as an amine-reactive dye. The fluorescein-labeled siRNA was dialyzed against water containing 0.1% diethylpyrocarbonate (DEPC) for 2 days in a dialysis membrane bag with a molecular weight (MW) cut-off of 3500, followed by lyophilization.

4.2. Synthesis of PEI-pullulan polymer

A mixture of 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) was stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature. After 4 h, 13.2 mg of linear polyethyleneimine (PEI; M_w, 22 kDa; 0.3 mmol) was added to the mixture and further stirred at room temperature under a nitrogen-rich atmosphere for 1 day. The mixture was dialyzed against water for 3 days in a dialysis Spectra Pore membrane bag with a molecular weight cut-off of 10,000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA), followed by lyophilization to obtain a PEI-pullulan polymer powder.

The buffering capacity of the PEI-pullulan polymer from pH 12 to 3 was determined by acid-base titration. Briefly, the polymer (4.8 mg) was dissolved in 8 mL of 150 mM NaCl to a final concentration of 0.6 mg/mL and the pH of the polymer solution was set to 12 with NaOH. The solution was subsequently titrated with 0.1 M HCl.

4.3. Measurements of the diameter of complexes

Polymer and siRNA complexes were prepared by incubating both the siRNA and the polymer in water for 30 min. The final concentration of the siRNA was adjusted to 1 μg/mL using water (pH 7.3). The diameters of the complexes were determined using a Zetasizer (Malvern Instruments, Malvern, UK) with the He/Ne laser at a detection angle of 173° and a temperature of 25 °C.

4.4. Electrophoresis of the polymer/siRNA complex

For the electrophoresis experiment, various concentrations of the polymer were mixed with the siRNA in ultrapure distilled water (Invitrogen) at room temperature for 30 min, and then analyzed by 19% polyacrylamide gel electrophoresis.

4.5. Delivery of polymer/siRNA complexes into mice by direct injection

All animal studies were performed in accordance with the Guidelines for Animal Experiments, established by the Ministry of Health, Labour and Welfare of Japan, and by the National Cardiovascular Center Research Institute. Male 6-week-old BALB/c mice (CLEA Japan Inc., Osaka, Japan) weighing approximately 22 g were used in this study. The mice were maintained in a temperature-controlled room (22 °C) with a 12-h light-dark cycle and were provided with a standard pellet diet (CE-2; CLEA Japan) and water ad libitum. One week after arrival, mice were divided into two groups, the hydrodynamics injection group and the non-hydrodynamics injection group. In the hydrodynamics injection group, 2 mL of 5% glucose solution containing each polymer/siRNA complex was injected, whereas in the non-hydrodynamics injection group, 0.2 mL was injected. For the hydrodynamics-based procedure, solutions were injected over 6–8 s into the tail vein using a 27-gauge needle. The mice were sacrificed 1 or 3 h after the injections, and thereafter each tissue type (lung, heart, liver, spleen, and kidney) was excised. Images were obtained with the Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Woburn, MA, USA).

Acknowledgment

This work was supported by a research grant from the Ministry of Health, Labour and Welfare (MHLW) and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan.

Supplementary data

Supplementary data (Tables S1 and S2 describing molecular parameters of polymer and zeta potential of PEI-pullulan/siRNA complex, respectively) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.031.

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Clinical Chemistry 56:3
478–481 (2010)

Brief Communications

C-Reactive Protein Uptake by Macrophage Cell Line via Class-A Scavenger Receptor

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BACKGROUND: C-reactive protein (CRP) increases in response to inflammation and is purported to be a risk factor for atherogenesis. We recently demonstrated that a scavenger receptor, lectin-like oxidized LDL receptor (LOX-1), is a receptor for CRP. In light of the overlapping ligand spectrum of scavenger receptors such as modified LDL, bacteria, and advanced glycation end products, we examined whether other scavenger receptors recognize CRP.

METHODS: We analyzed the uptake of fluorescently labeled CRP in COS-7 cells expressing a series of scavenger receptors and in a monocytic cell line, THP-1, differentiated into macrophage with phorbol 12-myristate 13-acetate (PMA). We applied small interfering RNA (siRNA) against class-A scavenger receptor (SR-A) to THP-1 cells to suppress the expression of SR-A. We also analyzed the binding of nonlabeled CRP to immobilized recombinant LOX-1 and SR-A in vitro using anti-CRP antibody.

RESULTS: COS-7 cells expressing LOX-1 and SR-A internalized fluorescently labeled CRP in a dose-dependent manner, but cells expressing CD36, SR-BI, or CD68 did not. The recombinant LOX-1 and SR-A proteins recognized nonlabeled purified CRP and native CRP in serum in vitro. THP-1 cells differentiated into macrophage-like cells by treatment with PMA-internalized fluorescently labeled CRP. siRNA against SR-A significantly and concomitantly inhibited the expression of SR-A ($P < 0.01$) and CRP uptake ($P < 0.01$), whereas control siRNA did not.

CONCLUSIONS: CRP is recognized by SR-A as well as LOX-1 and taken up via SR-A in a macrophage-like cell line. This process might be of significance in the pathogenesis of atherosclerotic disease.

C-reactive protein (CRP),[1] which is synthesized by hepatocytes in response to inflammation and tissue damage *(1)*, binds to various ligands exposed on damaged tissues or bacteria promoting phagocytosis and complement activation with C1q *(1, 2)*. Plasma CRP concentrations may rise as much as 1000-fold during infection or inflammation *(3)*. In addition, CRP concentrations, within the reference range, can predict cardiovascular diseases *(4, 5)*, and there is a good correlation between plasma CRP concentrations and the degree of atherosclerosis in hypercholesterolemic rabbits *(6)*.

Fcγ receptors CD16, CD32, and CD64 have been reported as the receptors for CRP *(7–9)*. In addition, we recently demonstrated that CRP increases vascular permeability through a direct binding to lectin-like oxidized LDL receptor (LOX-1), which is expressed in endothelial cells *(10)*. Members of the scavenger receptor family, such as class A scavenger receptor (SR-A), CD36, LOX-1, and scavenger receptor B-I (SR-BI), recognize common ligands such as modified LDL, bacteria, and advanced glycation end products, and they are thought to affect the progression of atherosclerosis *(11, 12)*. In this study, to further elucidate the atherogenic properties of CRP, we addressed whether other scavenger receptors are involved in the recognition of CRP.

Human sera with high and normal concentrations of CRP were obtained from Dako. Human CRP purified from pleural fluid was purchased from Chemicon (AG723). Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of Dulbecco's PBS (Wako). Gram-negative bacterial endotoxins were undetectable by limulus amebocyte lysate (Associates of Cape Cod), which can detect as little as 0.03 endotoxin units per mL endotoxins. CRP was fluorescently labeled with CypHer5E (GE Healthcare) and dialyzed 3 times against a 3000-fold volume of PBS.

COS-7 cells maintained with Dulbecco's modified Eagle's medium (DMEM; Invitrogen)/10% fetal bovine serum (FBS) were seeded 1 day before transfection. After reaching 80%–90% confluency, we transfected the cells with the plasmid using Lipofectamin 2000 transfection reagent (Invitrogen). We used the following cDNAs: human LOX-1 (GenBank NM002543), SR-A (GenBank NM002445), CD36 (GenBank NM000072), SR-BI (GenBank NM005505), CD68 (GenBank NM001251), and

[1] Nonstandard abbreviations: CRP, C-reactive protein; LOX-1, lectin-like oxidized LDL receptor; SR, scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; PMA, phorbol 12-myristate 13-acetate; SRA-C6, anti–SR-A antibody; DAPI, 4',6-diamidino-2-phenylindole.

dectin-1 (GenBank NM197947), which were subcloned into pcDNA6.2/V5/GW/D-TOPO expression vector (Invitrogen). We used pcDNA3.1/V5-His/lacZ (Invitrogen) as a control. After 48 h, we washed the cells with DMEM:1% antibiotics and antimycotic (AbAm; Invitrogen). We replaced the medium with CypHer5E-CRP-containing DMEM/1% AbAm and incubated the cells for 2 h at 37 °C. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the expression of each receptor by immunostaining with anti-V5 antibody (Nacalai Tesque) combined with Alexa 488 antimouse IgG (Invitrogen). The nuclei of the cells were counterstained with 0.5 mg/L 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field, then divided the CypHer5E-CRP fluorescence intensity in the field by the Alexa 488 fluorescence value. These quantitative analyses were performed with an IN Cell Analyzer 1000 system (GE Healthcare).

We prepared recombinant human SR-A (amino acids 76–358) as described for LOX-1 *(10)*. Recombinant human SR-A (0.1 μg) or BSA (0.1 μg, Sigma) was immobilized to each well of 384-well plates (High Bind; Corning) by incubating at 4 °C in PBS overnight. After 2 washes with PBS, the plates were blocked with 80 μL of 20% ImmunoBlock (DS Pharma)/PBS at 4 °C for 8 h. After washing twice with PBS, we added CRP in the reaction buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1% BSA, pH 7.0) to each well and incubated them at 4 °C overnight. We detected the binding of CRP with a TMB Peroxidase EIA Substrate kit (Bio-Rad) as described for LOX-1 *(10)*. We obtained small interfering RNA (siRNA) duplex oligoribonucleotides targeting the SR-A coding region (GenBank NM002445) from Invitrogen and used stealth RNAi duplex (Invitrogen) as a negative control. The siRNA sequences were as follows: 5′-GAUAUAACUCAAAGUCUCACGGGAA-3′, 5′-UUCCCGUGAGACUUUGAGUUAUAUC-3′ and 5′-CAGACCUUGAGAAAUAUCACUUUAA-3′, 5′-UUAAAGUGAUAUUUCUCAAGGUCUG-3′.

THP-1 cells were maintained with 10% FBS/1% AbAm/20 μmol/L mercaptoethanol:RPMI 1640 and differentiated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. We transfected the cells with siRNA oligos or control siRNA using Lipofectamin 2000 transfection reagent (Invitrogen) according to the manufacturer's instruction. After incubation at 37 °C for 24 h, we washed the cells with RPMI 1640/1% AbAm and replaced the medium with CypHer5E-CRP–containing RPMI 1640/1% AbAm, and the cells were incubated for 2 h. After washing with PBS, the cells were fixed with phosphate-buffered formalin (Wako) and permeabilized with 0.1% Triton X-100/PBS. We detected the effects of downregulation of SR-A gene expression by immunostaining with anti–SR-A antibody (SRA-C6; Trans Genic Inc) combined with Alexa 488 antimouse IgG. For detection of Fcγ receptors, we used anti-CD32 antibody (AT10; Santa Cruz) and anti-CD64 antibody (10.1; Santa Cruz). For CRP detection, we used anti-CRP antibody (Bethyl). The nuclei of the cells were counterstained with 0.5 mg/L DAPI. We divided the fluorescence intensities of CypHer5E and Alexa 488 by the cell number in a field. Quantitative analysis was performed with an IN Cell Analyzer 1000 system. All transfections were performed in triplicate.

All data are presented as mean (SE). Statistical analysis was performed with Student *t*-test. A P value <0.05 was considered statistically significant.

We examined whether CRP binds to scavenger receptors: LOX-1, SR-A, CD36, SR-BI, CD68, and dectin-1. Dectin-1 has the closest structural similarity to LOX-1 and belongs to C-type lectin-like molecule, although it is not a member of scavenger receptors.

Alexa546-labeled CRP at the concentration of 1 mg/L at 4 °C bound significantly to LOX-1–expressing cells ($P < 0.01$) but bound poorly to the cells expressing the other receptors (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue3). Cellular uptake of CypHer5E-labeled CRP, which shows fluorescence after endocytosis, was significantly higher in SR-A–expressing cells, in a dose-dependent manner (1–30 mg/L), as well as in LOX-1–expressing cells, compared with cells expressing the other receptors (Fig. 1). Immunostaining with anti-V5 antibody revealed that all the receptors were expressed at a similar level in the respective cells.

Using anti-CRP antibody, we confirmed that nonlabeled CRP was also taken up by SR-A–expressing COS-7 cells. We further observed a significant binding of nonlabeled CRP (0.1–1 mg/L) to immobilized recombinant SR-A ($P < 0.01$) (see online Supplemental Fig. 2). The binding was not affected by polymyxin B (5 mg/L), suggesting that it did not depend on the presence of endotoxin. Importantly, native CRP contained in human serum showed significant binding to SR-A, as well as to LOX-1 ($P < 0.01$) (see online Supplemental Fig. 3). The binding was dependent on the concentration of CRP in the serum, suggesting that SR-A and LOX-1 have a capacity to bind to a native form of CRP in serum in the presence of other plasma proteins. These results indicate that SR-A and LOX-1 are the receptors for CRP among the examined receptors.

Because SR-A works in the monocyte-macrophage system, we assessed whether CRP is taken up by macrophages via SR-A. We used a human monocytic cell line,

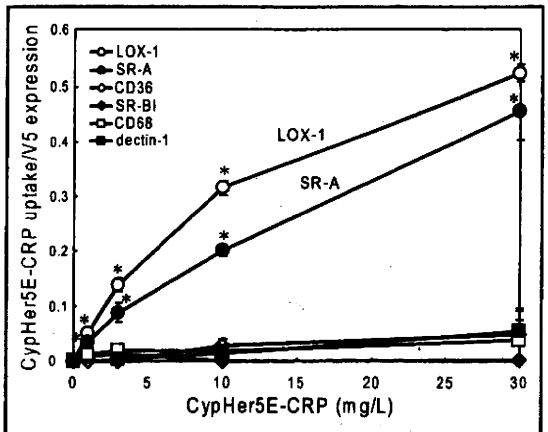

Fig. 1. Quantitative analyses of CypHer5E-CRP taken up by COS-7 cells expressing LOX-1, SR-A, CD36, SR-BI, and dectin-1.

Signals observed in the cells transfected with pcDNA3.1/V5-His/lacZ were considered as non-specific background. *Significant difference vs. negative control ($P < 0.01$).

Table 1. Suppression of the uptake of fluorescently labeled CRP by siRNA against SR-A in differentiated THP-1 cells.[a]

siRNA	SR-A expression, %	CypHer5E-CRP, %
None	103 (3.2)	108 (9.1)
Control siRNA	100 (2.7)	100 (9.8)
siRNA1 for SR-A	17 (1.6)[b]	31 (3.9)[b]
siRNA2 for SR-A	13 (0.0)[b]	32 (1.1)[b]

[a] Data are as mean (SE).
[b] Significant difference vs control siRNA groups ($P < 0.01$).

THP-1, after inducing differentiation into macrophage by the treatment of PMA *(13)*. In PMA-treated THP-1 cells, CypHer5E-CRP was taken up in a dose-dependent manner (0.3–30 mg/L). SR-A expression and CRP uptake were concomitantly suppressed by 2 different siRNAs targeting SR-A, but not by control siRNA (Table 1). The siRNA targeting SR-A did not affect the expression of Fcγ receptors (data not shown), indicating that CRP is taken up mainly via SR-A in a macrophage cell line, at least under these conditions.

The ligand specificity of the scavenger receptor family overlaps considerably *(11, 12)*, and while all can bind to oxidized LDL, only SR-A or LOX-1 bound to CRP. Interestingly, dectin-1, the most structurally similar molecule to LOX-1, did not bind to CRP.

Using a monoclonal antibody, a previous report suggested the presence of an unknown receptor other than Fcγ receptors in macrophages *(14)*. It has been reported that fucoidin, a ligand for SR-A, inhibits the in vivo CRP-promoted uptake of oxidized LDL *(15)*. SR-A might be the unidentified CRP receptor. Fcγ receptors and SR-A are under different regulation of gene expression. In fact, in response to differentiation stimulus of PMA, the expression of SR-A is strongly induced, whereas the expression of Fcγ receptors is suppressed *(16, 17)*. Conversely, stimulation by interferon-γ enhances the expression of Fcγ receptors but suppresses the expression of SR-A *(18)*. These results suggest that Fcγ receptors and SR-A would work in the cells stimulated by different molecules.

Interestingly, the activity of SR-A as CRP receptor was more pronounced in the uptake of CRP, whereas LOX-1 showed strong activity in both binding and uptake. Because SR-A works in phagocytes, the CRP uptake activity of SR-A is reasonable. CRP was originally identified as a binding protein for bacterial component C-polysaccharide *(3)*. SR-A may function to engulf bacteria, viruses, and harmful substances opsonized by CRP in a context of innate immunity.

Related to epidemiological risk factors for cardiovascular disease, the presence of CRP in atheroma has been reported in both rabbits and humans *(6)*. Furthermore, the colocalization of CRP and SR-A in macrophages in atheromas has been reported *(19)*. Taking these reports together with the present results, SR-A-mediated CRP uptake by macrophages in atheromas might affect the foam cell formation and progression of atherosclerotic disease.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of Health, Labour and Welfare of Japan; the National Institute of Biomedical Innovation; and Japan Science and Technology Agency.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at
DOI: 10.1373/clinchem.2009.140202

Original Article

Impact of Statin Treatment on the Clinical Fate of Heterozygous Familial Hypercholesterolemia

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Aim: **Familial hypercholesterolemia (FH) patients are at particular risk for premature coronary artery disease (CAD) caused by high levels of low density lipoprotein (LDL). Administration of statins enabled us to reduce LDL-C levels in heterozygous FH patients. To evaluate the impact of statins on the clinical fate of heterozygous FH, a retrospective study was performed.**
Methods: **We analyzed the clinical influence of statins on age at the first clinical onset of CAD in 329 consecutive FH patients referred to the lipid clinic of the National Cardiovascular Center. Among 329 heterozygous FH patients, the onset of CAD was identified in 101.**
Results: **The age at onset of CAD was 58.8 ± 12.5 years in the 25 patients on statins at onset, significantly higher than that in the 76 patients not on statins (47.6 ± 10.5 years) ($p<0.001$). The average age at CAD onset was significantly higher after widespread use of statins (54.2 ± 13.2 years in 48 patients; Group 1) compared to before October 1989 when statins were approved in Japan (46.9 ± 9.6 years in 53 patients; Group 2, $p=0.002$). A significant difference was seen between Groups 1 and 2 in the variables, including sex, prevalence of smoking habit, LDL-C, and the use of statins, aspirin and probucol. After adjusting for these variables, only statin use was independently associated with the difference in age at CAD onset by multivariable analysis.**
Conclusion: **Statins have improved the clinical course of patients with heterozygous FH.**

J Atheroscler Thromb, 2010; 17:667-674.

Key words; Familial hypercholesterolemia, Statin, Coronary artery disease, LDL cholesterol

Introduction

Familial hypercholesterolemia (FH) is a heritable disease of high prevalence with an autosomal-dominant mode of transmission and is linked to mutations in the low-density lipoprotein (LDL) receptor or its related gene. It is characterized by phenotypes of the elevation of plasma LDL, cutaneous and tendenous xanthomas, arcus corneae, and coronary artery disease (CAD) due to premature atherosclerosis[1]. The earliest clinical sign of heterozygous FH is an elevation of plasma LDL cholesterol (LDL-C), noted as early as at birth[2]. All other clinical manifestations seem due to an increase of LDL-C in plasma. CAD is the most serious clinical manifestation and determines the prognosis of FH. According to a previous report, Japanese FH heterozygotes generally develop the first CAD event in their 40s or later for men and 50s or later for

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Received: October 28, 2009
Accepted for publication: December 4, 2009

women[3].

To reduce plasma LDL-C in FH heterozygotes, bile acid-sequestering resins have been used since the 1970s to upregulate the LDL receptor, but their effect is limited to a 10 to 20% decline because of the concomitant induction of hepatic cholesterol synthesis[4]. Statins, competitive inhibitors of a rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, were introduced onto the market in the late 1980s. Pravastatin, the first approved statin in Japan, became commercially available at the beginning of October 1989 and simvastatin one year later[5]. Synthetic analogues became available in the late 1990s, including several "strong" statins, which lower the level of LDL-C by more than 40%[6]. Many large-scale clinical trials of statins worldwide, including Japan, showed that they reduced the risk of cardiac events or stroke in hypercholesterolemic populations[7-10]. Effective reduction of LDL-C by statins was also shown in FH heterozygotes[11, 12]; however, their clinical benefits in FH patients have not been clearly demonstrated with fixed clinical endpoints. This is partly because of the extremely high risk for CAD in FH patients, thus making controlled clinical trials of sufficient size to yield significant outcomes unethical.

Aim

Substantial numbers of FH patients have been referred to and regularly treated at the lipid clinic of the National Cardiovascular Center (NCVC) since it was founded in 1977. We therefore retrospectively analyzed the clinical records of these patients to assess the impact of the introduction of statins on the clinical prognosis of FH heterozygous patients, using patient age at the development of CAD. This parameter is specific and solid for each patient and the analysis is less influenced or biased by other factors. In addition, Mabuchi and colleagues used the same parameter in their study of Japanese FH reported before statin availability[13].

Methods

Subjects

Of the patients referred to the lipid clinic at NCVC from 1977 to 2007, 329 consecutive patients (139 men, 190 women) were diagnosed as FH heterozygotes using the criteria previously described[14]. Most of the FH patients analyzed in the present paper were referred to our lipid clinic by their general practitioner because of hypercholesterolemia. The medical records of patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2). Of the 329 FH patients, 101 were identified as having CAD, specifically, coronary artery stenosis (more than 75%) on angiography, including 53 patients who had CAD at the first clinic visit. The other 228 patients did not have clinical or angiographic evidence of CAD. For each patient, the age at onset of CAD was determined by the first sign, ascertained by a standardized questionnaire, which included fixed clinical endpoints of CAD, administered by attending physicians at the clinic. The compliance with statins was evaluated from the medical records.

Clinical Risk Factors

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or a blood pressure level higher than 140 mmHg systolic or 90 mmHg diastolic or both at the first clinic visit (the criteria for hypertension of the Japanese Society of Hypertension Guidelines)[15]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japan Diabetes Society[16]. A family history of CAD was identified by the standardized questionnaire. Smoking was identified from patients' self-reporting. Achilles tendon thickness was measured as previously described[17].

Analysis of Serum Lipids

Fasting plasma lipid concentration was measured before any lipid-lowering treatment. Total cholesterol (TC), triglycerides (TG), and HDL cholesterol (HDL-C) levels were measured enzymatically using an automated system in the clinical laboratory of the NCVC. LDL-C level was calculated by the Friedewald formula when the TG level was less than 400 mg/dL; three patients with TG level more than 400 mg/dL were omitted from this particular analysis. TG values were expressed as the median, (range), and logarithmically transformed before analysis.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 (SPSS Inc., Chicago, IL) program. Parametric values are expressed as the mean ± standard deviation (SD). The statistical significance of differences in continuous variables was evaluated by Student's t test for unpaired data or ANOVA. The Pearson's χ^2 test was used to assess differences in the distribution of categorical traits.

Table 1. Clinical characteristics of heterozygous FH patients with or without coronary artery disease (CAD) at first visit to our center.

	Total subjects	CAD (+)	CAD (−)	*p value*
n	329	101	228	
Age (years)	43.8±16.0	48.9±10.2	41.6±17.6	<0.001
Sex				
Men	139 (42.2%)	66 (65.3%)	73 (32.0%)	<0.001
BMI (kg/m^2)	22.0±3.2	23.0±2.7	22.6±3.3	<0.001
Total cholesterol (mg/dL)	319±70	333±85	313±61	0.039
Triglyceride (mg/dL)	(114) 80-176	(147) 96-193	(109) 76-162	0.263
HDL cholesterol (mg/dL)	50±17	42±14	54±17	<0.001
LDL cholesterol (mg/dL)	241±72	259±84	232±65	<0.001
Hypertension (*n*, %)	54 (16.4%)	33 (32.7%)	21 (9.2%)	<0.001
Diabetes Mellitus (*n*, %)	13 (4%)	8 (7.9%)	5 (2.2%)	0.014
Family history of CAD (*n*, %)	121 (36.8%)	46 (45.5%)	75 (32.9%)	0.028
Smoking habits (*n*, %)	127 (38.6%)	72 (71.3%)	55 (24.1%)	<0.001
Achilles tendon thickness (mm)	13.5±5.4	16.2±5.7	12.1±4.6	<0.001
CAD present at first visit (*n*, %)	53 (16.1)	53 (52.5)	0 (0)	<0.001
Statin treatment at first clinic visit	39 (11.9)	18 (17.8)	21 (9.2)	0.541

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.
BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; CAD, coronary artery disease

Results

Patient Background

The baseline clinical characteristics of the 329 heterozygous FH patients analyzed in this study are shown in **Table 1**. Their plasma lipid and lipoprotein profiles are similar to patients in previous reports of Japanese FH[3, 18]. Patients with CAD were older, had higher levels of BMI, TC, and LDL-C, lower HDL-C, and a higher incidence of diabetes mellitus, hypertension, a family history of CAD, and smoking habit, compared to patients without CAD.

Onset of CAD

In the 101 patients with CAD, age by decade at the first onset of CAD is illustrated in **Fig. 1**. The average age was 45.8±10.6 years in men and 59.0±9.5 years in women, and this is consistent with a previous report of Japanese FH patients[13]. Analysis of CAD onset in relation to the presence (+) or absence (−) of statin treatment showed that in the 66 FH men with CAD, 13 did and 53 did not have statin treatment, and in the 35 FH women with CAD, 12 did and 23 did not have statin treatment. The age distribution at the first onset of CAD in statin (+) or statin (−) patients is shown in **Fig. 2**. The peak was at an older age in statin (+) men and women (Panels A and B, respectively) compared to statin (−). The lipid profile at the time of first onset of CAD in statin (+) and statin (−)

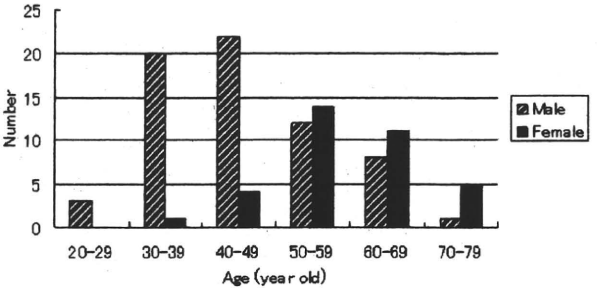

Fig. 1. Distribution of age when CAD was first identified in 101 men and women with heterozygous familial hypercholesterolemia (FH) and coronary artery disease (CAD), for the study period of 1969 to June 2007

patients is shown in **Table 2**. Statin (+) patients were older when CAD was identified and had lower TC and LDL-C levels than statin (−) patients.

To identify the factors that may influence the age at which CAD developed in statin (+) and statin (−) patients, we analyzed covariates (ANCOVA; **Table 3**), which included sex, smoking, BMI, hypertension, diabetes mellitus, family history of CAD, thickness of Achilles tendon, LDL-C levels, and the use of aspirin, probucol, and cholestyramine. We found that statin (+) patients were older when CAD developed, about 10 years older for each variable compared to statin (−) patients, which may be due to the use of statins and

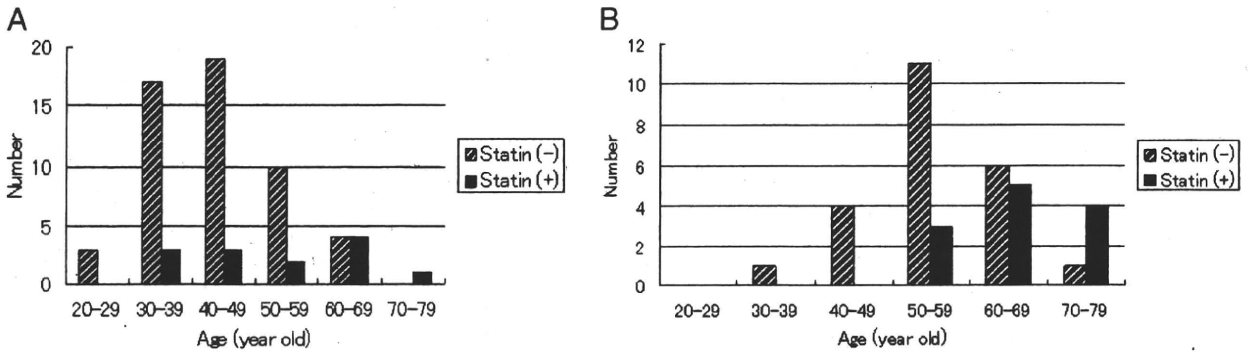

Fig. 2. Distribution of age when CAD was first identified in men (Panel A) and women (Panel B) with CAD taking a statin (+) or not (−)

Table 2. Age, lipid and lipoprotein profiles of FH at the onset of CAD in relation to statin use.

	Statin (+)	Statin (−)	*p value*
n	25	76	
Age of onset of CAD	57.8 ± 12.5	47.6 ± 10.5	<0.001
Lipid and lipoprotein profile at the event			
Total cholesterol (mg/dL)	242 ± 55	315 ± 108	<0.001
Triglycerides (mg/dL)	(127) 93-171	(115) 91-153	0.922
HDL cholesterol (mg/dL)	40 ± 12	38 ± 13	0.569
LDL cholesterol	167 ± 35	250 ± 108	<0.001

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.

Table 3. Onset age of CAD adjusted by each variable.

Variables	Age (95% CI) in Statin (+)	Age (95% CI) in Statin (−)	*p value*
Overall	57.8 (55.3-60.3)	47.6 (46.4-48.8)	<0.001
Smoking habit	58.2 (54.1-62.3)	47.3 (44.8-49.7)	<0.001
Sex	57.2 (53.3-61.0)	48.1 (45.9-50.3)	<0.001
BMI	58.9(54.4-63.3)	47.5 (45.0-50.1)	<0.001
Hypertension	59.4 (54.8-64.4)	47.4 (44.8-49.9)	<0.001
Diabetes mellitus	58.7 (54.3-63.1)	47.7 (45.2-50.3)	<0.001
Family history of CAD	58.8 (54.4-63.2)	47.1 (44.6-49.7)	<0.001
Achilles tendon thickness	58.7 (54.3-63.2)	46.7 (44.0-49.4)	<0.001
LDL cholesterol	58.4 (53.9-63.0)	47.6 (45.0-50.3)	<0.001
Aspirin	57.2 (52.9-61.5)	48.2 (45.7-50.7)	0.001
Probucol	56.0 (51.0-61.0)	48.6 (46.0-51.3)	0.017
Cholestyramine	58.2 (53.0-63.3)	47.9 (45.2-50.6)	0.001

the reduction of LDL-C.

To determine the impact of statin treatment on the age at which CAD developed, we analyzed the same data for the pre- and post-statin eras. Pravastatin was the first statin approved in Japan. Patients were divided into two groups: Group 1 developed CAD before the end of September 1989 (n=53) and Group 2 developed CAD from October 1989 (to June 2007; n=48). Of the 66 men with CAD, 39 were in Group 1 and 27 in Group 2, and of the 35 women with CAD, 14 were in Group 1 and 21 in Group 2. The men and women whose CAD developed after the beginning of October 1989 were older than those who developed CAD before that date (**Fig. 3A, B**). At the

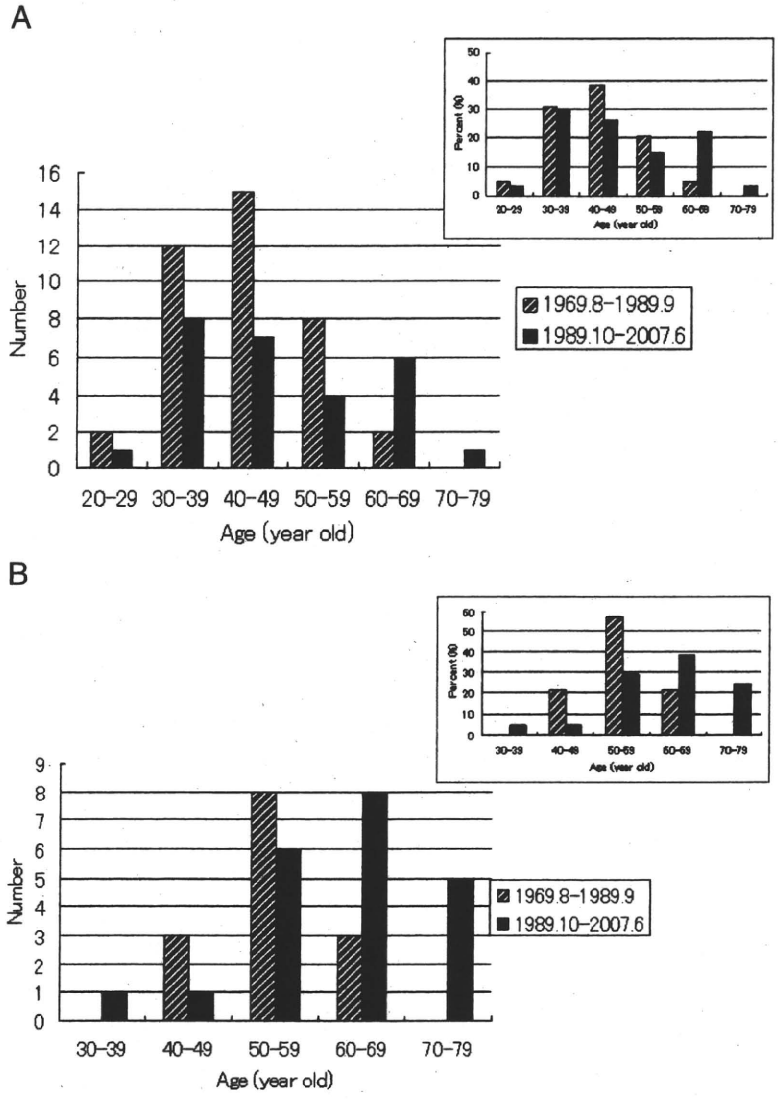

Fig. 3. Distribution of age at CAD onset in men (Panel A) and women (Panel B) who developed CAD before the end of September 1989 from October 1989

Each inset figure shows the percent of distribution, respectively.

first clinic visit, no clinical differences were seen in these patients in average age, BMI, plasma lipid and lipoprotein profile, Achilles tendon thickness and the incidence of hypertension, diabetes mellitus, and family history of CAD (**Table 4**); however, significantly more of the patients who developed CAD before the end of September 1989 were smokers. Assessment of clinical parameters obtained at the time CAD was identified shows that patients who developed CAD after the beginning of October 1989 were older (**Table 5**), reflecting the influence of statins on the onset age of CAD (**Fig. 3A, B**), and that TC and LDL-C levels were lower, reflecting that more of these patients were receiving lipid-lowering treatment than patients who developed CAD before this date.

Analysis of Factors that Affect Age at the First Onset of CAD

Age at the development of CAD in Groups 1 and 2 was analyzed using analysis of covariance (AN-

Table 4. Clinical characteristics (at first visit) of FH Patients depending on the onset date of CAD

	Group 1 1969–Sept. 1989	Group 2 Oct. 1989–June 2007	*p value*
n	53	48	
Age	48.4 ± 9.1	49.5 ± 11.4	0.584
Sex			
Male	39 (73%)	27 (56%)	0.068
BMI (kg/m^2)	22.6 ± 2.8	23.5 ± 2.6	0.288
Total cholesterol (mg/dL)	343 ± 84	321 ± 85	0.195
Triglycerides (mg/dL)	(114) 103–193	(148) 82–208	0.785
HDL cholesterol (mg/dL)	40 ± 15	44 ± 13	0.127
LDL cholesterol (mg/dL)	268 ± 80	250 ± 87	0.279
Hypertension (*n*, %)	21 (39.6%)	12 (25.0%)	0.118
Diabetes Mellitus (*n*, %)	2 (4%)	4 (8.3%)	0.535
Family history of CAD (*n*, %)	23 (43.4%)	25 (52.1%)	0.317
Smoking habits (*n*, %)	41 (83.7%)	31 (64.6%)	0.036
Achilles tendon thickness (mm)	16.0 ± 5.3	16.5 ± 6.1	0.710

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.

Table 5. Age, lipid and lipoprotein profiles and medication of FH at the onset of CAD.

	Group 1 1969–Sept. 1989	Group 2 Oct. 1989–June 2007	*p value*
n	53	48	
Age of onset of CAD	46.9 ± 9.6	54.2 ± 13.2	0.002
Lipid and lipoprotein profile at the event			
Total cholesterol (mg/dL)	323 ± 100	267 ± 95	0.011
Triglycerides (mg/dL)	(119) 96–162	(121) 79–152	0.427
HDL cholesterol (mg/dL)	36 ± 13	41 ± 12	0.088
LDL cholesterol	257±100	199 ± 95	0.011
Medication, *n* (%)			
Statin	1 (2.0)	24 (50.0)	< 0.0001
Probucol	6 (11.8)	17 (35.4)	0.005
Cholestyramine	3 (5.7)	11 (22.9)	0.015
Aspirin	1 (2.0)	7 (14.6)	0.021
No medication	44 (83.0)	22 (45.8)	< 0.001

Values are shown as Mean ± SD except for triglyceride. For triglyceride, median (range) is shown.

Table 6. Onset age of CAD adjusted by each variable.

Variables	Age (95% CI) in Group 1	Age (95% CI) in Group 2	*p value*
Overall	46.9 (44.2–50.0)	54.2 (50.3–58.0)	0.002
Smoking habits	46.9 (43.7–50.0)	53.4 (50.2–56.5	0.005
Sex	47.9 (45.2–50.7)	53.1 (50.2–55.9)	0.013
LDL cholesterol	48.2 (44.2–52.3)	54.5 (50.8–58.2)	0.029
Statin	49.1 (45.8–48.3)	51.8 (48.3–55.4)	0.325
Aspirin	47.9 (44.8–51.0)	53.2 (50.0–56.4)	0.021
Probucol	48.1 (45.0–51.2)	53.0 (49.8–56.2)	0.034
Cholestyramine	47.6 (44.4–50.8)	53.6 (50.2–56.9)	0.013

COVA; **Table 6**). Significant differences between groups were seen for sex, prevalence of smoking, LDL-C, and the use of statins, aspirin and probucol. After adjusting for these variables, statin use was independently associated with age at the onset of CAD.

Discussion

The mortality rate for CAD is 11 times higher in heterozygous FH patients than in the general population; thus, prevention of CAD is the key therapeutic goal for these patients[14]. Treatment to reduce high levels of LDL-C in FH patients was limited before statins became available, and a clinically meaningful decrease in LDL-C levels was difficult to obtain. Pravastatin was first introduced onto the Japanese market at the beginning of October 1989 and thereafter, LDL-C reductions of 20% to 30%, even in FH heterozygous patients, became possible[19]. Recently, the risk of myocardial infarction in heterozygous FH was reported to be reduced by 76%, similar to the general population of the Netherlands[20]. In the present paper, we assessed the impact of statin use on the clinical prognosis of Japanese FH patients visiting our lipid clinic by retrospectively analyzing their clinical records. The use of statins delayed the first CAD event by about 7 years in FH patients whose first event occurred after the introduction of statins, compared to FH patients whose first event occurred prior to the introduction of statins.

In this study, 101 of 329 (30.6%) consecutive heterozygotes of FH had clinical evidence of CAD. The profile of CAD patients is similar to previous reports, that is, more men than women[3, 21, 22], and higher BMI, higher TC and LDL-C levels, lower HDL-C levels, and a higher incidence of hypertension, diabetes mellitus, family history of CAD, and smoking[3, 13, 23, 24].

The time span of our study allowed us to assess the impact on the development of CAD of the introduction of statins onto the Japanese market at the beginning of October 1989. Comparing clinical parameters at the first clinic visit in the patients whose CAD developed before the end of September 1989 with after that date, revealed that only smoking was different, perhaps reflecting the social trend against smoking (**Table 4**). In contrast, interesting differences between these groups were seen in relation to when they developed CAD. Patients who developed CAD prior to the introduction of statins were younger on average (46.9 years old) and had higher levels of TC and LDL-C (323 and 257 mg/dL, respectively). Two other prominent differences were the improved lipid-lowering drug regimens, including statins, cholestyramine, probucol, and aspirin, and a decline in the number of smokers. Notably, statin use was independently and significantly associated with age at CAD onset in the 101 FH patients on covariate analysis of factors known to affect the age of developing CAD. Besides these factors, many other factors should be considered for the potential influence on the onset age of CAD, such as the widespread recognition of FH and the regimen for the treatment of other risk factors, such as hypertension and diabetes mellitus. Nevertheless, we should conclude from this analysis that the use of statins is a major factor contributing to the improvement of the clinical prognosis of FH patients in Japan.

More recently, "strong" statins have become available, making it possible to reduce LDL-C levels to much lower levels compared to conventional statins in FH patients[25-27]. The possible impact of these stronger statins on delaying the development of CAD in FH patients will be of interest.

One diagnostic criterion for heterozygous FH in the existing guidelines is a family history of premature CAD[28-30]. However, our results suggest that this criterion may need to be reconsidered because of the proven ability of statin treatment to delay the development of CAD to an age similar to that in persons who do not have heterozygous FH.

We showed in this retrospective analysis that the development of CAD was delayed by about 7 years in FH patients whose CAD developed after the introduction of statins in Japan compared to those whose CAD developed before the current statin era.

Acknowledgments

This work was supported by the Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labour and Welfare (H20-genomu-008 and H20-nanji-ippan-011) and the Cardiovascular Research Foundation (Suita, Japan).

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〈連　載〉
症例検討
脂質代謝異常症への多角的アプローチ
96

PCSK9遺伝子変異とLDL受容体遺伝子変異を合併したホモ接合体性家族性高コレステロール血症の一例

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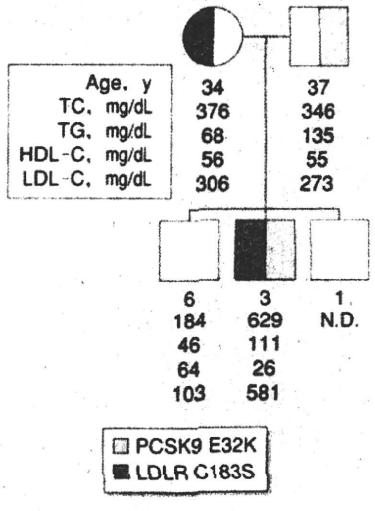
はじめに

日常臨床で遭遇する高コレステロール血症は，動脈硬化症，特に冠動脈硬化症の主な原因疾患である．原発性高脂血症の多くは遺伝要因を基盤として発症すると考えられ，単一遺伝子疾患で最も重症な高脂血症は家族性高コレステロール血症（familial hypercholesterolemia；FH）である．

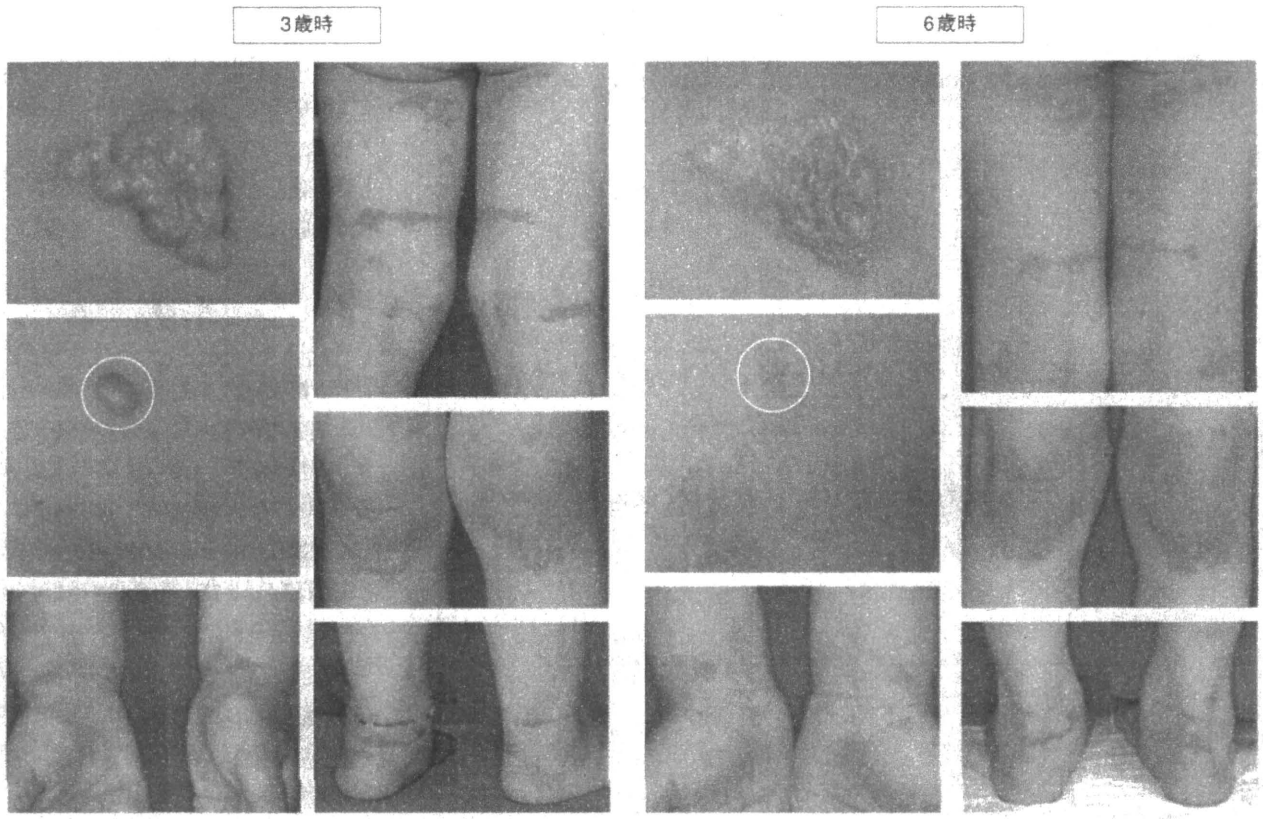
FHは，臨床的に高LDLコレステロール（LDL-C）血症，腱黄色腫，および早発性冠動脈硬化症を3主徴とする常染色体優性遺伝性疾患である．ヘテロ接合体性FHは一般人口500人に1人以上と，高頻度に存在すると考えられている．FHは主としてLDL受容体（LDLR）遺伝子異常が原因であり，われわれの検討では，FHと臨床診断された症例の62.5％がLDLR遺伝子変異を有していた[1]．また，LDLRに対するリガンドであるアポリポ蛋白B（アポB）の遺伝子変異を原因とするfamilial defective apolipoprotein B-100（FDB）も，FHの臨床像を呈する．東欧ではFHと診断された患者の10％程度と高頻度であるが[2]，わが国では存在しないかきわめて低頻度であることをわれわれは報告している[3]．

近年，LDLR，アポBに加え，proprotein convertase subtilisin/kexin type 9（PCSK9）遺伝子変異もFH様症状の病因となることが注目されている．PCSK9はセリンプロテアーゼの一種で，肝臓や小腸などから血中に分泌され，主に肝臓のLDLR分解を亢進して細胞膜表面のLDLR量を低下させることで，血中のLDL C濃度に影響を与える．そのため，機能亢進型変異では高脂血症[4]，機能低下型変異では低脂血症[5]を呈する．わが国においては，機能亢進型PCSK9 E32K変異が遺伝性高脂血症の原因として高頻度に存在することをわれわれは報告した[6]．欧米で報告されているPCSK9 D374Yなどの機能亢進型PCSK9変異はFHと同等の脂質値を示すが[7]，PCSK9 E32K変異ではFHに比して臨床像が軽症であった．

本稿では，ホモ接合体性FHの臨床像を呈する症例の中からわれわれが見出した，PCSK9 E32K変異とLDLR遺伝子変異のダブルヘテロ接合体症例の臨床所見および治療効果について，文献的考察を加えて報告する．



図❶　家系図および脂質プロファイル



図❷ 本症例の黄色腫

症 例

症例：3歳の男児（紹介受診当時）．

家族歴：両親に血族結婚なし（図❶，家系図参照）．

病歴：2003年7月（満1歳2ヵ月），手関節および足関節に黄色腫を認めたため，近医を受診した．総コレステロール（TC）629mg/dL，トリグリセライド（TG）111mg/dL，HDLコレステロール（HDL-C）26mg/dL，LDL-C 581mg/dLと著明な高LDL-C血症が認められ，臨床的にホモ接合体性FHと診断された．3歳時に精査加療目的に当科に紹介受診となった．

身体所見（紹介受診当時）：身長99.5cm（+0.7 SD），体重14.3kg（−0.2 SD）．眼瞼黄色腫なし．角膜輪なし．頸部リンパ節腫脹なし．甲状腺腫なし．両手首，両足首，両肘，両膝，両大腿屈側などに広がる全身性の皮膚黄色腫あり（図❷）．

一般検査成績：AST 27IU/L，ALT 12IU/L，γ-GTP 10IU/L，LDH248IU/L，BUN 12mg/dL，Cr 0.22mg/dL，CK 147IU/L，ALP1,018IU/L，WBC 7,800/μL，RBC 447×10^4/μL，Hb 11.8g/dL，Ht 35.2%，Plt 23.0×10^4/μL，TC 599mg/dL，TG 61mg/dL，HDL-C 33mg/dL，LDL-C 519mg/dL，apo AI 90mg/dL，AⅡ23mg/dL，B 277mg/dL，CⅡ3.3mg/dL，CⅢ 7.0mg/dL，E 6.9mg/dL，Lp