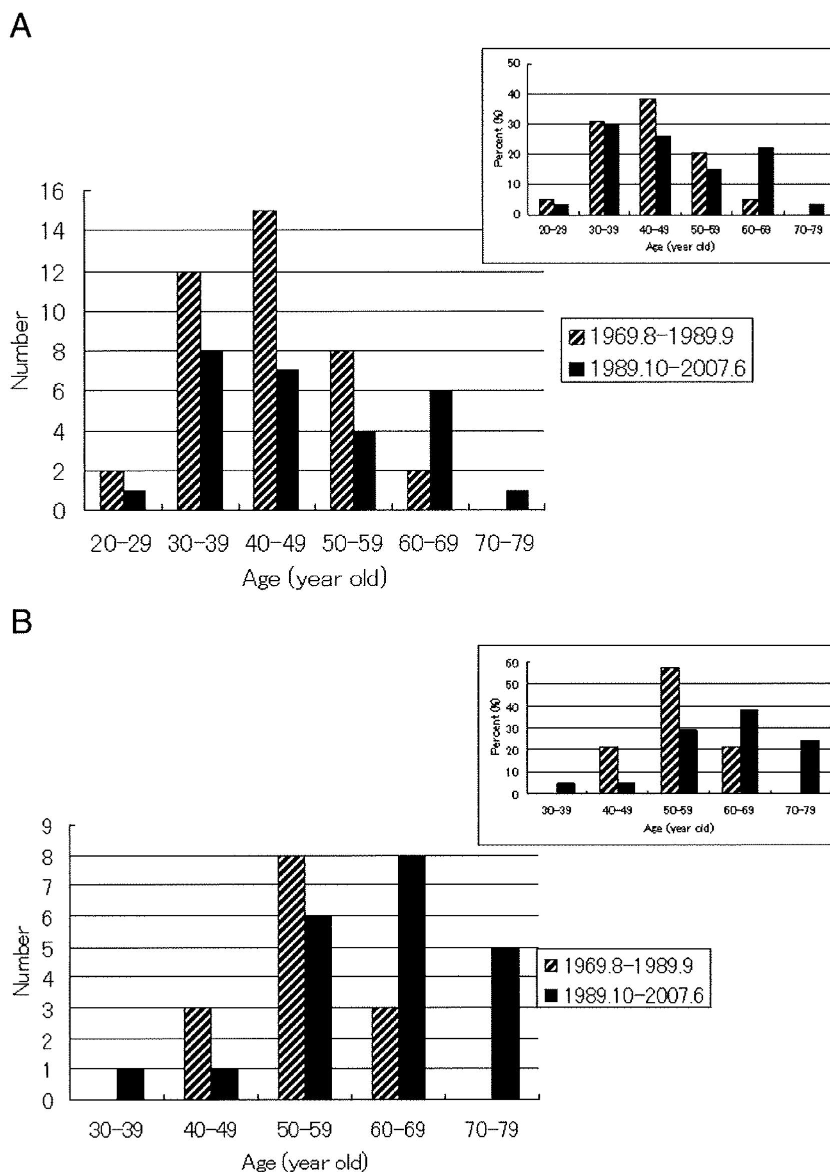


Fig. 3. Distribution of age at CAD onset in men (Panel A) and women (Panel B) who developed CAD before the end of September 1989 from October 1989

Each inset figure shows the percent of distribution, respectively.

ily 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 which CAD was identified shows that patients who developed CAD from 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 (ANCOVA; **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 inde-

4143(2)

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

4143(2)

pendently 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 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 (47.6 years old) and had higher levels of TC and LDL-C (6.2 and 4.3 mmol/L, 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).

References

1) Goldstein JL HH, Brown MS, Familial hypercholesterolemia, New York, McGraw-Hill, 2001: 2863-2913
2) Kwiterovich PO, Jr., Levy RI, Fredrickson DS: Neonatal diagnosis of familial type-II hyperlipoproteinaemia. Lancet, 1973; 1: 118-121
3) Bujo H, Takahashi K, Saito Y, Maruyama T, Yamashita S, Matsuzawa Y, Ishibashi S, Shionoiri F, Yamada N, Kita T: Clinical features of familial hypercholesterolemia in Japan in a database from 1996-1998 by the research committee of the ministry of health, labour and welfare of Japan. J Atheroscler Thromb, 2004; 11: 146-151
4) Simons LA, Williams PF: Changes in plasma lipoproteins in subjects treated with the bile acid-sequestering resin polidexide (Secholex). Aust N Z J Med, 1976; 6: 127-130
5) Illingworth DR, Bacon S: Hypolipidemic effects of HMG-CoA reductase inhibitors in patients with hypercholesterolemia. Am J Cardiol, 1987; 60: 33G-42G

4143(2)

6) Naoumova RP, Marais AD, Mountney J, Firth JC, Rendell NB, Taylor GW, Thompson GR: Plasma mevalonic acid, an index of cholesterol synthesis in vivo, and responsiveness to HMG-CoA reductase inhibitors in familial hypercholesterolaemia. Atherosclerosis, 1996; 119: 203-213
7) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. N Engl J Med, 1998; 339: 1349-1357
8) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet, 1994; 344: 1383-1389
9) Nakamura H, Arakawa K, Itakura H, Kitabatake A, Goto Y, Toyota T, Nakaya N, Nishimoto S, Muranaka M, Yamamoto A, Mizuno K, Ohashi Y: Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA Study): a prospective randomised controlled trial. Lancet, 2006; 368: 1155-1163
10) Amarenco P, Goldstein LB, Szarek M, Sillesen H, Rudolph AE, Callahan A, 3rd, Hennerici M, Simunovic L, Zivin JA, Welch KM: Effects of intense low-density lipoprotein cholesterol reduction in patients with stroke or transient ischemic attack: the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial. Stroke, 2007; 38: 3198-3204
11) Mabuchi H, Haba T, Tatami R, Miyamoto S, Sakai Y, Wakasugi T, Watanabe A, Koizumi J, Takeda R: Effect of an inhibitor of 3-hydroxy-3-methyglutaryl coenzyme A reductase on serum lipoproteins and ubiquinone-10-levels in patients with familial hypercholesterolemia. N Engl J Med, 1981; 305: 478-482
12) Naoumova RP, Dunn S, Rallidis L, Abu-Muhana O, Neuwirth C, Rendell NB, Taylor GW, Thompson GR: Prolonged inhibition of cholesterol synthesis explains the efficacy of atorvastatin. J Lipid Res, 1997; 38: 1496-1500
13) Mabuchi H, Koizumi J, Shimizu M, Takeda R: Development of coronary heart disease in familial hypercholesterolemia. Circulation, 1989; 79: 225-232
14) Mabuchi H, Miyamoto S, Ueda K, Oota M, Takegoshi T, Wakasugi T , Takeda R: Causes of death in patients with familial hypercholesterolemia. Atherosclerosis, 1986; 61: 1-6
15) Ikeda N, Hasegawa T, Hasegawa T, Saito I, Saruta T: Awareness of the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2000) and compliance to its recommendations: surveys in 2000 and 2004. J Hum Hypertens, 2006; 20: 263-266
16) Matsushima M: [Japan Diabetes Society clinical practice guideline]. Nippon Rinsho, 2002; 60 Suppl 9: 161-166
17) Mabuchi H, Tatami R, Haba T, Ueda K, Ueda R, Ito S, Karnetani T, Koizurni J, Miyamoto S, Ohta M, Takeda R, Takegoshi T, Takeshita H: Achilles tendon thickness and ischemic heart disease in familial hypercholesterolemia. Metabolism, 1978; 27: 1672-1679
18) Mabuchi H, Tatami R, Ueda K, Ueda R, Haba T, Kametani T, Watanabe A, Wakasugi T, Ito S, Koizumi J, Ohta M, Miyamoto S, Takeda R: Serum lipid and lipoprotein levels in Japanese patients with familial hypercholesterolemia. Atherosclerosis, 1979; 32: 435-444
19) Mabuchi H, Kamon N, Fujita H, Michishita I, Takeda M, Kajinami K, Itoh H, Wakasugi T, Takeda R: Effects of CS-514 on serum lipoprotein lipid and apolipoprotein levels in patients with familial hypercholesterolemia. Metabolism, 1987; 36: 475-479
20) Versmissen J, Oosterveer DM, Yazdanpanah M, Defesche JC, Basart DC, Liem AH, Heeringa J, Witteman JC, Lansberg PJ, Kastelein JJ, Sijbrands EJ: Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. Bmj, 2008; 337: a2423
21) Heiberg A: The risk of atherosclerotic vascular disease in subjects with xanthomatosis. Acta Med Scand, 1975; 198: 249-261
22) Beaumont V, Jacotot B, Beaumont JL: Ischaemic disease in men and women with familial hypercholesterolaemia and xanthomatosis. A comparative study of genetic and environmental factors in 274 heterozygous cases. Atherosclerosis, 1976; 24: 441-450
23) Hirobe K, Matsuzawa Y, Ishikawa K, Tarui S, Yamamoto A, Nambu S, Fujimoto K: Coronary artery disease in heterozygous familial hypercholesterolemia. Atherosclerosis, 1982; 44: 201-210
24) Vuorio AF, Turtola H, Piilahti KM, Repo P, Kanninen T, Kontula K: Familial hypercholesterolemia in the Finnish north Karelia. A molecular, clinical, and genealogical study. Arterioscler Thromb Vasc Biol, 1997; 17: 3127-3138
25) Marais AD, Firth JC, Bateman ME, Byrnes P, Martens C, Mountney J: Atorvastatin: an effective lipid-modifying agent in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol, 1997; 17: 1527-1531
26) Kajinami K, Koizumi J, Ueda K, Miyamoto S, Takegoshi T, Mabuchi H: Effects of NK-104, a new hydroxymethylglutaryl-coenzyme reductase inhibitor, on low-density lipoprotein cholesterol in heterozygous familial hypercholesterolemia. Hokuriku NK-104 Study Group. Am J Cardiol, 2000; 85: 178-183
27) Stein EA, Strutt K, Southworth H, Diggle PJ, Miller E: Comparison of rosuvastatin versus atorvastatin in patients with heterozygous familial hypercholesterolemia. Am J Cardiol, 2003; 92: 1287-1293
28) Sullivan D: Guidelines for the diagnosis and management of familial hypercholesterolaemia. Heart Lung Circ, 2007; 16: 25-27
29) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Primary hyperlipidemia. J Atheroscler Thromb, 2008; 15: 49-51
30) Civeira F: Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. Atherosclerosis, 2004; 173: 55-68

4143(2)

Papers in Press. Published January 14, 2010 as doi:10.1373/clinchem.2009.140202
The latest version is at http://www.clinchem.org/cgi/doi/10.1373/clinchem.2009.140202

Clinical Chemistry 56:3
000–000 (2010)

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

Yoshiko Fujita, Akemi Kakino, Mariko Harada-Shiba, Yuko Sato, Kazunori Otsui, Ryo Yoshimoto, and Tatsuya Sawamura*

Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka, Japan; *address correspondence to this author at: Department of Vascular Physiology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Fax +81-6-6835–5329; e-mail t-sawamura@umin.ac.jp.

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.

Copyright (C) 2010 by The American Association for Clinical Chemistry

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 (Supplemental Fig. 1, which accompanies the online version of this article at 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 ($P < 0.01$) (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 ($P < 0.01$) (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,

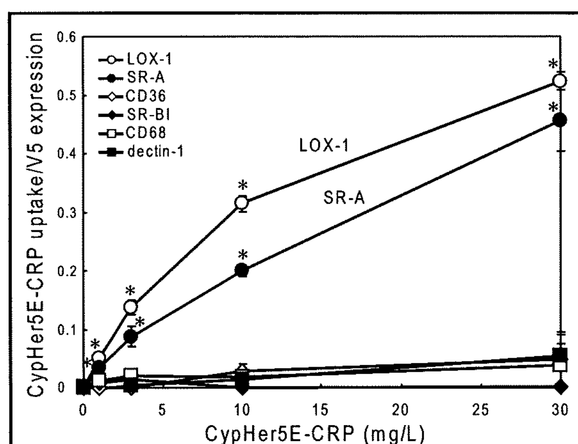


Fig. 1. Quantitative analyses of CypHer5E-CRP taken up by COS-7 cells expressing LOX-1, SR-A, CD36, SR-BI, and dectin-1.

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

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.

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 ($P < 0.01$).

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: This study was supported in part by 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.

References

1. Black S, Kushner I, Samols D. C-reactive protein. J Biol Chem 2004;279:48487–90.
2. Volanakis JE, Kaplan MH. Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. J Immunol 1974;113:9–17.
3. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999;340:448–54.
4. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336:973–9.
5. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008; 359:2195–207.
6. Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, et al. C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. Am J Pathol 2005;167: 1139–48.
7. Khreiss T, Jozsef L, Hossain S, Chan JS, Potempa LA, Filep JG. Loss of pentameric symmetry of C-reactive protein is associated with delayed apoptosis of human neutrophils. J Biol Chem 2002; 277:40775–81.
8. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. The major receptor for C-reactive protein on leukocytes is Fcgamma receptor II. J Exp Med 1999;190:585–90.
9. Stein MP, Mold C, Du Clos TW. C-reactive protein binding to murine leukocytes requires Fc gamma receptors. J Immunol 2000;164:1514–20.
10. Fujita Y, Kakino A, Nishimichi N, Yamaguchi S, Sato Y, Machida S, et al. Oxidized LDL receptor LOX-1 binds to C-reactive protein and mediates its vascular effects. Clin Chem 2009;55:285–94.
11. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol 2006;26:1702–11.
12. Greaves DR, Gordon S. The immune system and atherogenesis: recent insights into the biology of macrophage scavenger receptors. Thematic review series. J Lipid Res 2005;46:11–20.
13. Nishimura N, Harada-Shiba M, Tajima S, Sugano R, Yamamura T, Qiang QZ, Yamamoto A. Acquisition of secretion of transforming growth factor-beta 1 leads to autonomous suppression of scavenger receptor activity in a monocyte-macrophage cell line, THP-1. J Biol Chem 1998; 273:1562–7.
14. Tebo JM, Mortensen RF. Characterization and isolation of a C-reactive protein receptor from the human monocytic cell line U-937. J Immunol 1990;144:231–8.
15. Singh U, Dasu MR, Yancey PG, Afify A, Devaraj S, Jialal I. Human C-reactive protein promotes oxidized low density lipoprotein uptake and matrix metalloproteinase-9 release in Wistar rats. J Lipid Res 2008;49:1015–23.
16. Fleit HB, Kobasiuk CD. The human monocyte-like cell line THP-1 expresses Fc gamma RI and Fc gamma RII. J Leukoc Biol 1991;49:556–65.
17. Morganelli PM, Kennedy SM, Mitchell TI. Differential effects of interferon-gamma on metabolism of lipoprotein immune complexes mediated by specific human macrophage Fcgamma receptors. J Lipid Res 2000;41:405–15.
18. Geng YJ, Hansson GK. Interferon-gamma inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages. J Clin Invest 1992;89:1322–30.
19. Turk JR, Carroll JA, Laughlin MH, Thomas TR, Casati J, Bowles DK, Sturek M. C-reactive protein correlates with macrophage accumulation in coronary arteries of hypercholesterolemic pigs. J Appl Physiol 2003;95:1301–4.

Previously published online at
DOI: 10.1373/clinchem.2009.140202

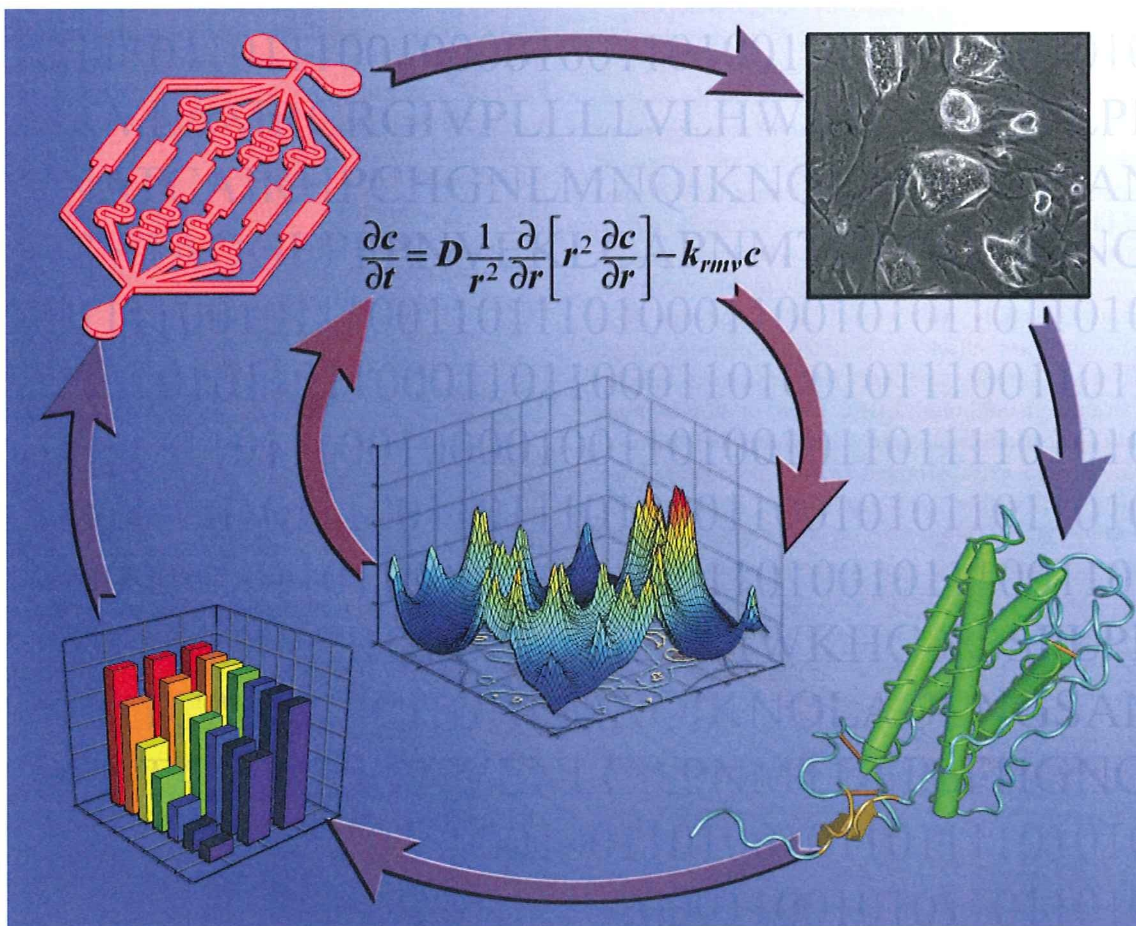
Molecular BioSystems

This article was published as part of the

2009 Emerging Investigators Issue

Highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces

Please take a look at the full table of contents to access the other papers in this issue.



In vivo siRNA delivery with dendritic poly(L-lysine) for the treatment of hypercholesterolemia†‡

Kazuto Watanabe,[a] Mariko Harada-Shiba,[b] Akira Suzuki,[b] Risa Gokuden,[a] Ryohsuke Kurihara,[a] Yusuke Sugao,[a] Takeshi Mori,[ac] Yoshiki Katayama[ac] and Takuro Niidome*[acd]

Received 30th January 2009, Accepted 12th August 2009
First published as an Advance Article on the web 21st August 2009
DOI: 10.1039/b900880b

Intravenous delivery of apolipoprotein B-specific siRNA with a sixth-generation of dendritic poly(L-lysine) (KG6) resulted in siRNA-mediated knockdown of ApoB in healthy C57BL/6 mice without hepatotoxicity, and with a significant reduction of serum low-density lipoprotein cholesterol in apolipoprotein E-deficient mice.

siRNA-mediated specific gene silencing has generated great interest in its use as a research tool and as a therapeutic agent for a wide spectrum of disorders that include cancer, infectious disease, and metabolic conditions. Effective *in vivo* siRNA delivery is essential for siRNA-based applications and a variety of nonviral and viral systems are being developed.[1]

Apolipoprotein B (ApoB) is an essential protein for the formation and secretion of very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) in the metabolism of dietary and endogenous cholesterol. ApoB is expressed in hepatocytes. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease (CAD), and ApoB and LDL-cholesterol (LDLc) levels are suggested to be controlled in patients with a high risk of CAD. Statins, a class of drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are currently widely used to reduce this risk. Although statins are effective cholesterol-lowering drugs, two-thirds of statin-treated patients still experience adverse coronary events. In addition, LDLc levels of homozygous familial hypercholesterolemia can't be controlled by oral administration, and extracorporeal treatment is needed for these patients. Therefore, new alternative strategies are needed.[2] ApoB is too large a protein to collect the 3D structural data; accordingly, it is not a suitable target for conventional small-molecule drug development. Therefore, as an alternative strategy, ApoB silencing by RNAi is quite promising.

Once *in vivo*, intravenous delivery of cholesterol-conjugated siRNA against ApoB (si–ApoB) and subsequent reduction of serum LDLc had been achieved,[3,4] *in vivo* ApoB knockdown experiments were reported, using various siRNA delivery systems. Lipid-based systems, known as stable nucleic acid–lipid particles (SNALP) and interfering nanoparticles (iNOP), have increased nuclease stability and decreased the injection dose of siRNA to clinically relevant levels.[5,6] A polymer-based system called Dynamic PolyConjugates, which is functionalized with a *N*-acetyl-galactosamine ligand for hepatocyte targeting and linked to siRNA with a disulfide bond for reductive release, offers efficient and nontoxic delivery of siRNA.[7] Recently, a new class of lipid-like delivery agents, termed lipidoids, was synthesized and evaluated for safe and effective *in vivo* siRNA delivery.[8] The efficiency to deliver siRNA to hepatocytes was evaluated based on the changes of ApoB mRNA levels after injection of si–ApoB formulated in various lipidoid nanoparticles. Thus si–ApoB is not only a new therapeutic agent for hypercholesterolemia, but also a research tool available for characterization of newly developed hepatocyte-targeting carriers in terms of transfection efficiency.

We previously reported that a sixth generation of dendritic poly(L-lysine) (KG6, Fig. 1),[9] consists of amino acid, and has advantages in having a monodispersed structure that can be synthesized using easy protocols. It has a high plasmid DNA transfection ability with low cytotoxicity *in vitro*.[9–12] The efficiency was comparable to commercially available transfection reagents such as Lipofectin, JetPEI, and Superfect. In particular, during plasmid DNA transfection, the addition of chloroquine, which enhances endosome escape to the cytosol, was not required, and serum could be added to the medium during transfection. It can be concluded that KG6 is a simple and highly efficient transfection reagent like the commercially available transfection reagents that work *in vitro*. KG6 can be also used as a siRNA carrier into cells. After adding the siRNA complex of KG6 to cultivated cells, efficient uptake of the siRNA into the cells was observed with low cytotoxicity. The efficiency was higher and the cytotoxicity was lower than those of Lipofectamine 2000, a commercially available transfection reagent.[13] We recently evaluated the performance of this promising molecule, KG6, as a gene carrier *in vivo*.[14] We investigated the biodistribution of plasmid DNA delivered with KG6 in mice after intravenous administration. Southern blotting analysis revealed that more than 20% of the plasmid

[a] *Department of Applied Chemistry, Faculty of Engineering, Kyushu University, Fukuoka 819-0395, Japan. E-mail: niidome.takuro.655@m.kyushu-u.ac.jp; Fax: +81-92-802-2851; Tel: +81-92-802-2851*
[b] *Department of Bioscience, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan*
[c] *Center for Future Chemistry, Kyushu University, Fukuoka 819-0395, Japan*
[d] *PRESTO, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan*

† This article is part of a *Molecular BioSystems* 'Emerging Investigators' issue highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces.
‡ Electronic supplementary information (ESI) available: Supplementary tables and figures. See DOI: 10.1039/b900880b

 This journal is © The Royal Society of Chemistry 2009

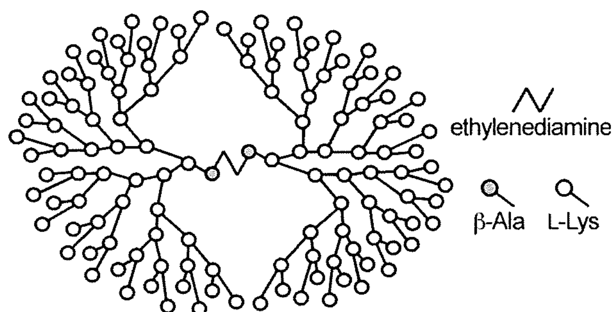


Fig. 1 The structure of the sixth-generation dendritic poly(L-lysine) (KG6).

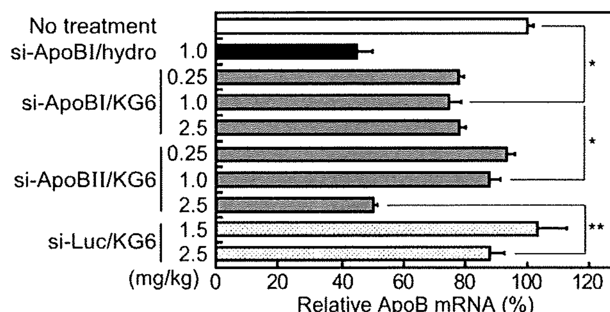


Fig. 2 Reduction of ApoB mRNA levels in the liver treated with the siRNA complexes of KG6. RT-qPCR of liver ApoB levels relative to GAPDH was performed 24 h after injection of si–ApoBI, si–ApoBII or si–Luc with KG6 as a carrier (siRNA/KG6), or by hydrodynamic-based administration as a positive control (siRNA/hydro). Data were normalized to untreated mice. Significances of differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$). Data are means ± S.E. ($n > 3$).

DNA had accumulated in the liver and 10% of the DNA complexes with KG6 at a cation/anion (C/A) ratio of 8.0 remained circulating in the blood for 30 min after intravenous injection. Almost all of the DNA circulating in the blood would also be trapped in the liver and then gradually degraded. Thus the hepatotropic accumulation of intact plasmid DNA is one of the noteworthy characteristics of KG6. Based on these characteristics, efficient siRNA delivery to hepatocytes should be possible using KG6.

We started with siRNA complex of KG6 at C/A ratio of 8.0 formed in 5% dextrose, and the particle size of the complex was determined by dynamic light scattering. The size was 168 ± 9.9 nm (Table S1, ESI‡). We had previously confirmed that the size of the pDNA complex of KG6 at the C/A ratio of 8.0 was 238 ± 5.2 nm.[9] In addition, we determined the electrophoretic mobilities of the siRNA complexes of KG6 at different C/A ratios using 20% native polyacrylamide gel electrophoresis in TBE buffer at pH 8.0. No migration of the siRNA band occurred at a C/A ratio of 1.0 or above (Fig. S1‡). For the KG6 pDNA complex, electrophoresis retardation occurred at the same C/A ratio.[9] Therefore, the characteristics of the siRNA complex of KG6 were expected to be similar to those of the pDNA complex, and hepatotropic accumulation of the siRNA complex after intravenous injection was anticipated.

We next determined the ability of KG6 to deliver siRNA to hepatocytes and to silence ApoB expression *in vivo*. The si–ApoBI complex, si–ApoBII complex, and luciferase-specific siRNA (si–Luc) complex as a control, were delivered to C57BL/6 mice by intravenous injection. Livers from injected mice were harvested 24 h after the injection and assayed for ApoB mRNA levels by reverse transcriptase quantitative PCR (RT-qPCR). The ApoB mRNA levels were relative to the level of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. As shown in Fig. 2, the ApoB mRNA levels in mice treated with the si–ApoBI complex were reduced by approximately 22% compared with those in the no-treatment group, at all of the doses evaluated. si–ApoBII complex showed significant reduction of the mRNA. Especially, a 50% reduction was observed at 2.5 mg kg^{-1} dose, and it was a significantly stronger effect compared with the case of si–Luc (2.5 mg kg^{-1}), which showed a slight reduction.

The reduced levels of ApoB mRNA mediated by si–ApoBI were lower than those previously reported using the SNALP and iNOP systems (approximately 77% and 50% reduction, respectively).[5,6] In the case of si–ApoBII, approximately 87% reduction was observed by using Dynamic PolyConjugates.[7] The difference in efficiencies between our KG6 system and their systems may be due to the chemical structure of the siRNA. In their systems, the siRNAs contained a 2′-*O*-methyl (2′-OMe), 2′-fluoro modification (2′-F) or a phosphorothioate (PS) linkage.[5–7] In general, chemical modification of siRNA not only improves stability but also reduces off-target effects.[15] Furthermore, appropriate modifications do not reduce the siRNA activity and the 2′-OMe modification, in particular, is calculated to maintain siRNA activity by retaining the canonical right-handed A-form helical geometry required for RNAi. Here, we adopted to deliver unmodified siRNAs using KG6. Therefore, it is considered that our less active and stable si–ApoB would result in the lower efficiency of ApoB knockdown than that achieved using the lipid- and polymer-based systems mentioned above, and si–Luc, as a negative control, might nonspecifically interfere in its expression in a dose-dependent manner.[16,17]

The other reason is that the ability of KG6 to deliver siRNA to hepatocytes might be insufficient. In fact, ApoB mRNA levels reduced by 55% in mice administered with only 1.0 mg kg^{-1} of chemically unmodified si–ApoBI by a hydrodynamic injection, which is a positive control procedure to deliver an siRNA gene or oligonucleotide to hepatocytes.[18] The insufficient delivery with KG6 could be attributed to the size of the complex. It has been suggested that complexes of > 100–200 nm would be restricted by the fenestrations in liver blood vessels and thus unable to access the hepatocytes.[19] As the particle size of siRNA complex of KG6 is larger than 100 nm (Table S1, ESI‡), some of the complexes would be unable to cross the fenestrations and become trapped in the Kupffer cells.

The potential toxicity of the KG6 siRNA complex was assessed by measuring the serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are two enzymes located in liver cells and which leak into the circulation when liver cells are injured. Slight elevations of AST and ALT activities were detected in mice treated with 2.5 mg kg^{-1} of the KG6 siRNA complex compared with untreated mice at 24 h after injection. The levels of AST and

This journal is © The Royal Society of Chemistry 2009

ALT (IU L^{-1}) were as follows: 19.4 ± 1.2 and 6.1 ± 0.5 respectively, in untreated mice, and 22.7 ± 1.1 and 10.7 ± 0.4, respectively, in mice injected with the complex. Because the increased levels were not significant, the intravenous delivery of siRNA with KG6 appears to be well tolerated.

Finally, improvement of hypercholesterolemia was observed after treating apolipoprotein E (ApoE)-deficient ($ApoE^{-/-}$) mice with the KG6 siRNA complex. ApoE is a ligand for cell-surface lipoprotein receptors such as the LDL-receptor (LDLr) and LDLr-related proteins (LRP). Homozygous deletion of the *ApoE* gene in mice results in a pronounced increase in the plasma levels of LDL and VLDL, which is attributable to the failure of LDLr- and LRP-mediated clearance of these lipoproteins.[20] $ApoE^{-/-}$ mice are one of the most widely used hypercholesterolemic mouse models. In ApoB knockdown experiments, it was reported that intraperitoneal administration of a mouse-specific ApoB antisense oligonucleotide, ASO ISIS 147764, to $ApoE^{-/-}$ mice reduced the plasma levels of LDLc by 40%.[21] Here, we focused on si–ApoBI, which matches with not only mouse ApoB mRNA but also human's, while si–ApoBII has a mismatch with human ApoB mRNA.[3,7] We intravenously injected $ApoE^{-/-}$ mice (weighing 30–40 g) with 50 μg of the KG6 si–ApoBI complex. A single dose of the si–ApoBI complex resulted in a decrease in VLDLc and LDLc levels for up to 96 h, whereas no decrease in their levels was observed with the 5% dextrose only or the si–Luc complex (Fig. 3). Change of total serum cholesterol was similar to that of VLDLc and LDLc (data not shown). We also determined the ApoB mRNA levels 96 h after injection and found that the ApoB mRNA levels remained reduced only in the si–ApoBI-treated group (reduction in ApoB mRNA levels: 24.6 ± 2.6% *vs.* 7.6 ± 9.4%, respectively). These results indicate that systemic siRNA delivery with KG6 could provide a clinically useful; approach to reducing cholesterol levels in patients with hypercholesterolemia.

In summary, our results show that KG6 is a promising carrier to deliver siRNA to silence endogenous genes in clinically acceptable doses and improve hypercholesterolemia. In prior reports in which si–ApoB was delivered *in vivo*, a reduction in serum total cholesterol levels in healthy C57BL/6 mice was observed.[3–7] However, mice lack the cholesterol ester transferase protein (CETP) and carry the majority of their plasma cholesterol as HDL. Because plasma lipoprotein compositions in $ApoE^{-/-}$ mice resemble those in patients with hypercholesterolemia more closely than in C57BL/6 mice, our ApoB knockdown experiment with siRNA in $ApoE^{-/-}$ mice is valuable for assessing RNAi therapeutics to target hypercholesterolemia. For clinical use of KG6, however, further investigations of the KG6 complex in terms of its physical properties and physiological effects are needed. Recently, it was suggested that an electrostatically formed complex of siRNA differs from pDNA in terms of encapsulation efficiency, stability and other physical properties.[22] As we have investigated the biodistribution of plasmid DNA delivered with KG6, an assessment of the difference between plasmid DNA and siRNA should be performed.

Our results also indicate that KG6 is applicable for intravenous delivery of other types of therapeutic oligonucleotides such as antisense and decoy oligonucleotides to the liver.

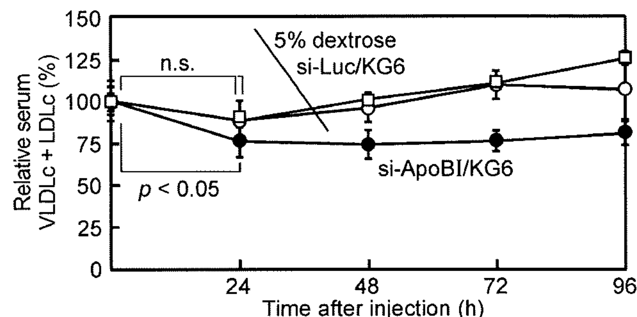


Fig. 3 Therapeutic effect of the KG6 si–ApoBI complex in a mouse model of hypercholesterolemia. $ApoE^{-/-}$ mice (30–40 g) were injected intravenously with 50 μg of the si–ApoBI complex of KG6 (closed circles), si–Luc complex (open circles) or 5% dextrose only (open squares) ($n > 3$, respectively). Serum was obtained from 6-hour-fasted mice every 24 h after administration for determination of LDLc and VLDLc levels. Values represent the rate of change in each cholesterol level. Data are shown as means ± S.E. Significances of differences from each group before treatment are indicated. n.s. indicates no significance.

In addition, precise functionalization of the surface primary amino groups can be achieved by modification with hydrophilic polymer such as a polyethylene glycol for stable circulation in blood flow,[23] glycation and peptide ligand modification for site-specific delivery.[7,24] Thus KG6 is expected to be a base molecule for efficient and targeted nucleotide delivery *in vivo*.

Experimental

Chemicals and instruments

Organic solvents used in all synthetic procedures and RNA extraction, ethylenediamine and transaminase CII-test Wako were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*-Boc-protected β-alanine and lysine were purchased from Novabiochem, Merck Ltd. (Tokyo, Japan). The coupling reagents, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazol (HOBt) were purchased from Watanabe Chemical (Hiroshima, Japan). Trifluoroacetic acid (TFA) was purchased from Kanto Chemical (Tokyo, Japan). siRNAs were purchased from Gene Design Inc. (Osaka, Japan). RNA iso Plus, SYBR PrimeScript RT-PCR Kit II (Perfect Real Time), and primers for RT-qPCR were purchased from Takara Bio Inc. (Shiga, Japan). The particle sizes of the KG6 siRNA complexes were measured with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom). RT-qPCR was performed with a LightCycler ST300 (Roche Diagnostics, Basel, Switzerland). Plasma lipoproteins were analyzed by high-performance liquid chromatography (HPLC) using molecular sieve columns, Skylight Biotech, Inc. (Akita, Japan).

siRNAs

The siRNAs had the following sequences: si–ApoBI, sense 5′-GUCAUCACACUGAAUACCAAU-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGACAC-3′;[3–5] si–ApoBII, sense 5′-GAAUGUGGGUGGCAACUUUAG-3′, antisense 5′-AAAGUUGCCACCCACAUUCAG-3′;[7] si–Luc (GL-3 luciferase

This journal is © The Royal Society of Chemistry 2009

reporter gene), sense 5′-CUUACGCUGAGUACUUCGAUU-3′, antisense 5′-UCGAAGUACUCAGCGUAAGUU-3′.[7]

Synthesis of dendritic poly(L-lysine)

Dendritic poly(L-lysine) was synthesized as previously described.[7] In brief, for the initial core synthesis, *N*-Boc-protected β-alanines were coupled with ethylenediamine in DMF by the HBTU-HOBt method, and deprotection was then performed by TFA treatment. For the synthesis of the first and higher generations, the coupling reaction between the amino group-free previous generation of dendrimers and *N*-Boc-protected lysines was performed in DMF by the HBTU–HOBt method, and Boc-groups were then removed by TFA. We synthesized dendrimers up to the sixth generation (KG6) by repetition of these coupling and deprotection procedures. The molecular weights of these synthesized dendrimers were measured by MALDI-TOF-MS (data not shown).

Preparation of the siRNA complex

For administration of appropriate amounts of siRNA (if we consider the amount as X μg), X μl of 1.0 μg μl^{-1} siRNA was added to 60 μl of 25% dextrose. After the addition of (240 − 2X) μl of water, X μl of 0.2 mM KG6·128TFA salt solution was added to the mixture and incubated for 10 min at room temperature to form KG6 siRNA complexes at a C/A ratio of 8.0. The C/A ratio means a molar ratio of cationic amino groups (KG6)/anionic phosphate groups (siRNA). All aqueous solutions were prepared with DEPC-treated water.

Animals

Male C57BL/6N mice (7-weeks old, 19–21 g) were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). Male $ApoE^{-/-}$ mice on C57BL/6J background mice (8-weeks old) were obtained from Jackson Laboratories (Bar Harbor, Me., USA). All animal experiments were carried out in accordance with Guidelines for the Animal care and Use committee, Kyushu University and the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Mice were housed in a room at 24 ± 2 °C with a 12 h light–dark cycle before being used in the experiments. Food and water were available *ad libitum*.

In vivo siRNA administration procedure

For intravenous administration, 300 μl of KG6 siRNA complexes at a C/A ratio of 8.0 in 5% dextrose was injected *via* the tail vein over 5 s. For hydrodynamics-based administration, which is an established method for *in vivo* gene or oligonucleotide transfer to mouse liver,[16] mice were injected *via* the tail vein with a volume equivalent to 10% of the body weight within 5 s.

Liver harvest, RNA isolation and RT-qPCR assay

Twenty-four hours after injection, mice were sacrificed and perfused *via* the portal vein before the liver was harvested. Total RNA was isolated from the liver immediately after harvest using RNA iso Plus and reverse transcribed using the PrimeScript RT Reagent Kit according to the manufacturer's protocol. Relative quantification of the target gene mRNA compared with the housekeeping gene GAPDH mRNA was determined by quantitative PCR assay using SYBR Premix Taq II and gene-specific primers on a LightCycler ST300 according to the manufacturer's protocol.

Hepatotoxicity analysis

Three hundred μl of KG6 siRNA complexes at a C/A ratio of 8.0 in 5% dextrose was administered *via* the tail vein to 7-week-old male C57BL/6N mice. Blood was collected 24 h after administration. Serum was prepared by incubation of collected blood for 30 min at room temperature and centrifugation at 2000 × *g* for 20 min at 4 °C. The serum AST and ALT activities were measured using a transaminase CII-test Wako.

Determination of plasma lipoprotein compositions

Three hundred μl of the complexes containing 50 μg of siRNA at a C/A ratio of 8.0 in 5% dextrose was administered *via* the tail vein to $ApoE^{-/-}$ mice (31, 34, 40 or 52 weeks old, 30–40 g). Mice were starved for 6 h and blood was collected from a tail vein every 24 h after administration. Serum HDL or LDL/VLDL cholesterol levels were measured in each blood sample, analyzed by HPLC.[25]

Statistical analysis

Treatment effects were analyzed by one-way ANOVA. For statistically significant F-values, means were compared by Fisher's multiple range tests. All data described in context also follow this analytic method and are shown as means ± S.E.

Acknowledgements

This research was supported by a Grant-in-Aid for Exploratory Research (No. 18650124) and a Grant-in-Aid for Scientific Research (B) (No. 19300172) from the Japan Society for the Promotion of Science (JSPS).

References

1 T. Nguyen, E. M. Menocal, J. Harborth and J. H. Fruehauf, *Curr. Opin. Mol. Ther.*, 2008, **10**, 158–167.
2 C. P. Cannon, E. Braunwald, C. H. McCabe, D. J. Rader, J. L. Rouleau, R. Belder, S. V. Joyal, K. A. Hill, M. A. Pfeffer and A. M. Skene, *N. Engl. J. Med.*, 2004, **350**, 1495–1504.
3 J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H.-P. Vornlocher, *Nature*, 2004, **432**, 173–178.
4 C. Wolfrum, S. Shi, K. N. Jayaprakash, M. Jayaraman, G. Wang, R. K. Pandey, K. G. Rajeev, T. Nakayama, K. Charrise, E. M. Ndungo, T. Zimmermann, V. Koteliansky, M. Manoharan and M. Stoffel, *Nat. Biotechnol.*, 2007, **25**, 1149–1157.
5 T. S. Zimmermann, A. C. H. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L. V. Nechev, L. R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.-P. Vornlocher and I. MacLachlan, *Nature*, 2006, **441**, 111–114.

This journal is © The Royal Society of Chemistry 2009

6 H. Baigude, J. McCarroll, C. Yang, P. M. Swain and T. M. Rana, *ACS Chem. Biol.*, 2007, **2**, 237–241.
7 D. B. Rozema, D. L. Lewis, D. H. Wakefield, S. C. Wong, J. J. Klein, P. L. Roesch, S. L. Bertin, T. W. Reppen, Q. Chu, A. V. Blokhin, J. E. Hagstrom and J. A. Wolff, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 12982–12987.
8 A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. Fougerolles, J. R. Dorkin, K. N. Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Y. Sah, J. Soutschek, I. Toudjarska, H.-P. Vornlocher, T. S. Zimmermann, R. Langer and D. G. Anderson, *Nat. Biotechnol.*, 2008, **26**, 561–569.
9 M. Ohsaki, T. Okuda, A. Wada, T. Hirayama, T. Niidome and H. Aoyagi, *Bioconjugate Chem.*, 2002, **13**, 510–517.
10 T. Okuda, S. Kidoaki, M. Ohsaki, Y. Koyama, K. Yoshikawa, T. Niidome and H. Aoyagi, *Org. Biomol. Chem.*, 2003, **1**, 1270–1273.
11 T. Okuda, A. Sugiyama, T. Niidome and H. Aoyagi, *Biomaterials*, 2004, **25**, 537–544.
12 M. Yamagata, T. Kawano, K. Shiba, T. Mori, Y. Katayama and T. Niidome, *Bioorg. Med. Chem.*, 2007, **15**, 526–532.
13 Y. Inoue, R. Kurihara, A. Tsuchida, M. Hasegawa, T. Nagashima, T. Mori, T. Niidome, Y. Katayama and O. Okitsu, *J. Controlled Release*, 2008, **126**, 59–66.
14 T. Kawano, T. Okuda, H. Aoyagi and T. Niidome, *J. Controlled Release*, 2004, **99**, 329–337.
15 D. de Paula, M. V. L. B. Bentley and R. I. Mahato, *RNA*, 2007, **13**, 431–456.
16 S. P. Persengiev, X. Zhu and M. R. Green, *RNA*, 2004, **10**, 12–18.
17 D. Semizarov, L. Frost, A. Sarthy, P. Kroeger, D. N. Halbert and S. W. Fesik, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 6347–6352.
18 E. Song, S. K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar and J. Lieberman, *Nat. Med.*, 2003, **9**, 347–351.
19 S. Kawakami and M. Hashida, *Drug Metab. Pharmacokinet.*, 2007, **22**, 142–151.
20 S. Zadelaar, R. Kleemann, L. Verschuren, J. V.-V. Weij, J. Hoorn, H. M. Princen and T. Kooistra, *Arterioscler., Thromb., Vasc. Biol.*, 2007, **27**, 1706–1721.
21 R. M. Crooke, M. J. Graham, K. M. Lemonidis, C. P. Whipple, S. Koo and R. J. Perera, *J. Lipid Res.*, 2005, **46**, 872–884.
22 S. Spagnou, A. D. Miller and M. Keller, *Biochemistry*, 2004, **43**, 13348–13356.
23 S. H. Kim, J. H. Jeong, S. H. Lee, S. W. Kim and T. G. Park, *J. Controlled Release*, 2008, **129**, 107–116.
24 R. M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P. Y. Lu, P. V. Scaria and M. C. Woodle, *Nucleic Acids Res.*, 2004, **32**, e149.
25 M. Harada-Shiba, A. Takagi, K. Marutsuka, S. Moriguchi, H. Yagyu, S. Ishibashi, Y. Asada and S. Yokoyama, *Circ. Res.*, 2004, **95**, 945–952.

This journal is © The Royal Society of Chemistry 2009

第2章 先端科学技術とDDS開発 ①

医用工学とDDS

DDS for Biomedical Engineering

国立循環器病センター研究所 先進医工学センター 生体工学部
山岡哲二
TETSUJI YAMAOKA
Department of Biomedical Engineering, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute

はじめに

医用工学に関する多くの学会では，人工臓器や生体材料のセッションに加えてDDS関連のセッションが設けられている。DDS研究は，工学者にとっても魅力的な領域であり，いろいろなDDS用材料群や新たなシステムが開発研究されてきた。医薬品は，広義には「身体の構造または機能に影響を及ぼすことが目的とされているものであって，機械器具などではないもの」であり，医療機器と区別されているようであるが，近年，これらは急激に接近して，その境界はもはや明確ではない。また，分子生物学・再生医工学の進歩とともに，さまざまなタンパク製剤やホルモン製剤，あるいはさまざまな生体由来シグナル分子が医薬品となり，さらに，機能細胞までもが「身体の機能に影響をおよぼすもの」として捉えられる時代が始まった。本稿では，医療機器研究の立場から，DDSあるいは医薬品との今後の関わりについて考えたい。

1．機能性医療機器

薬事法は，医薬品だけではなく，医薬部外品，化粧品，そして，医療機器も規制している。昭和23年に作られた「新たな薬事法」から長い年月を経て，平成17年に改正薬事法が実施された。それに伴い，医療用具は医療機器と改名されている。一口に医療機器と言っても，医療現場で使うピンセット，注射針からMRI，そして，マッサージチェアまでが，薬事法で規制される医療機器である。医療機器開発は，医薬品開発・DDS研究と協同的に歩んできた。非分解性の人工弁・人工心臓・人工血管などの抗血栓性は完全なものではなく，医薬品投与とDDS技術導入によりサポートされ，また，生体活性分子を用いた表面修飾によっても，さらなる機能化が施されてきた（**図1**）。近年，薬物放出ステント（DES：Drug-Eluting Stent）に代表されるように直接的に融合した医療機器の出現に伴い，両者の境界はますます見えにくくなっている。

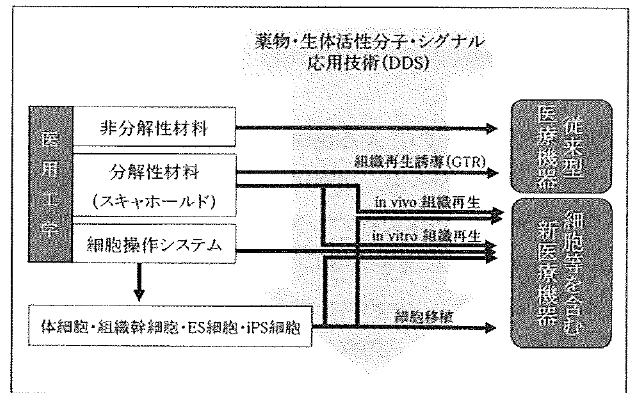


図1　医薬品・DDSに大きくサポートされる医用工学研究

(1)ステント

ステント適応後の再狭窄防止を目的に，再狭窄抑止効果のある薬物を徐放するDESは，2004年に輸入承認されて国内販売が始まった。同年のDESの市場は約160億円で，翌年には約600億円にまで達した[1]。現在のDESには，

主に，細胞増殖抑制を作用機序とするパクリタキセル系，および，免疫抑制を主な作用機序とするシロリムス系が用いられている。システムとしてはDDSデバイスのようにも感じられるが，承認は医療機器であり，融合デバイスの象徴的存在である。近年の，ステントの全市場が900億円程度であることから，その中のDESのシェアは75％にものぼる。一方で，2006年米国心臓学会で，遅発性ステント血栓症（LST：Late Stent Thrombosis）の発症率がDES適応例で有意に高いことが示され，現在も，その有効性と安全性が議論されていることもあり，このシェアに動きが出始めている。LSTのメカニズムはいまだ解明されていないが，薬物放出担体としてステント表面にコーティングされているマトリックス材料に対する長期炎症のために，一度構築された内皮層が遅発性に損傷を受けることによるとの危惧もあり，新規材料に対する研究開発が精力的に進んでいる。まさに研究開発においても，医療機器開発とDDS研究との融合が必須である。

(2)生理活性分子による医療機器の機能化

同様の目的のために，2005年には，表面に抗CD34抗体をコーティングしたステントの臨床研究成果が発表された[2]。末梢血中に存在するCD34陽性細胞である血管内皮前駆細胞（EPC：Endotherial Progenitor Cell[3]）がステント表面に結合して，内皮化促進につながることを狙った新規デバイスである。さらに，翌年には，インテグリン接着性細胞接着因子であるRGDトリペプチドをコーティングしたステントにおいてもEPCのリクルートメントが誘導できるとの臨床研究が報告されている[4]。抗CD34抗体もRGDトリペプチドも，まさに「身体の機能に影響をおよぼすもの」として捉えることができる。

われわれは，再生型人工血管の臨床研究で優れた実績を有する東京女子医科大学の研究グループ[5]と共同で，ポリ乳酸／ポリカプロラクトン共重合体多孔質人工血管に対する抗CD34抗体の固定化を検討してきた[6]。すなわち，抗CD34抗体修飾した生体吸収性人工血管に対してCD34陽性の造血幹細胞あるいはEPCをリクルートする治療システムの構築を目指した（図2）。すでに，東京女子医大にて血管再生用スキャホールドとして用いられているポリ乳酸の多孔質体の表面を，所定濃度のNaOHで加水分解することで表面にカルボキシル基を導入し，カルボジイミド法により，抗CD34抗体を固定化した。この抗体修飾スキャホールド上に，CD34陽性細胞，CD34陰性細胞，および，イヌ骨髄細胞を約2×10^6個/50mlの濃度で，流速0.05ml/minで播種し，接着した細胞数を計数して免疫染色にて評価したところ，抗体固定化スキャホールド上には，陽性／陰性混合細胞系では80％程度のCD34陽性細胞濃縮効率が得られ，イヌ骨髄細胞播種システムでは約4倍のCD34陽性細胞が認められた。このような，生理活性分子により修飾された医療機器の機能性は，生理活性分子の固定化や徐放化と大きく関連し，人工骨や人工皮膚，神経誘導管など多くの再生医療用医療機器で今後検討が進むと考えられ得る。

2．再生医療の進歩とともに

米国において始まったTissue Engineering（組織工学）研究は，大きな損傷を受けた組織や臓器を修復でき，さらに，組織移植や人工臓器の欠点を補う新たな治療法として注目された。1993年，R. Langerらは，スキャホールド（Scaffold，足場材料）と呼ばれたポリグリコール酸

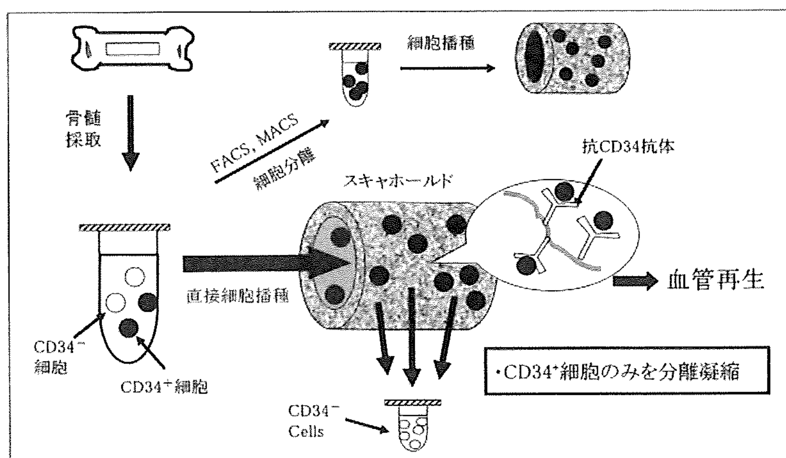


図2　幹細胞特異的抗体を表面に固定化した血管再建用スキャホールド

(PGA)の不織布に軟骨細胞を播種してヌードマウスの皮下に埋入することで，異所的な軟骨の再生が誘導できることを示唆した[7]。その後，わが国においては，2007年10月にヒト自家移植組織(ジェイス™)が，わが国初のヒト細胞を含む医療機器としての製造承認を得た[8]。実は，このようなマトリックスと細胞とを融合させるアイデアは古くから検討され，基礎技術の確立から30年の時を経ての実用化である。これらの新たな医療機器においては，今後も，薬物や細胞とマトリックスとの融合技術がボトルネックとなる(図1)。

細胞を用いた医療機器開発において，機能細胞の安定な調製は困難を極めたが，1981年にマウス胚性幹細胞が[9,10]，さらに1998年には，ヒト胚性幹細胞の単離が報告され[11]，allogeneicな体細胞の入手が可能になると期待された。その後，2007年には山中らがヒトiPS細胞(induced Pluripotent Stem Cell)を発表することで，autologousな体細胞の入手までもが可能な状況となりつつある。そのため，最も単純かつ，早期の臨床化が期待される再生医療の系として，細胞移植療法が精力的に検討され，心筋梗塞[12,13]や下肢虚血，筋萎縮性側索硬化症[14]に対する基礎検討から，臨床成果に至る多くの報告がなされている。このような状況のもと，組織の治癒力を利用した組織再生に直接的に作用すると期待されるのが増殖因子[15]であり，さまざまな投与システムによる効果増強に期待がもたれる[16]。ここでは，変遷を続ける今後の再生医療を支援するための物質送達システムのわれわれの取り組みについて2点紹介したい。

(1)インジェクタブルスキャホールド

これまで述べてきたように，従来の薬物のみならず，タンパク質やシグナル分子，さらには，細胞やその集合体としての小さな組織までが「身体の機能に影響をおよぼすもの」として利用され始め，今後のDDSは，薬物を送達するのみならず，細胞や組織をも送達する必要がある。例えば，幹細胞の懸濁液を直接心筋組織に注入した場合には，細胞の生着率は極めて低い。そこで，細胞注入を支援する材料として，体内で，水溶液から含水ゲルへ相転移する生体吸収性材料(インジェクタブルスキャホールド)が注目されている。2003年バイオマテリアル誌に，"Injectable Polymeric Material" という特集が組まれ，体内でゲル化する材料の有用性が提唱された。当時，光反応性基や，化学反応性基，あるいは，ポリ(N-イソプロピルアクリルアミド)などの温度応答性ポリマーが利用されたが，いずれもその生体内での安全性は確保されていない。われわれは，京都工芸繊維大学木村良晴教授らと共同で研究を進め[17]，PLAとポリエチレングリコール(PEG)という，生体内での利用実績に優れる2つの高分子材料のみを利用することで，温度応答性を実現させインジェクタブルスキャホールドとすることに成功した。

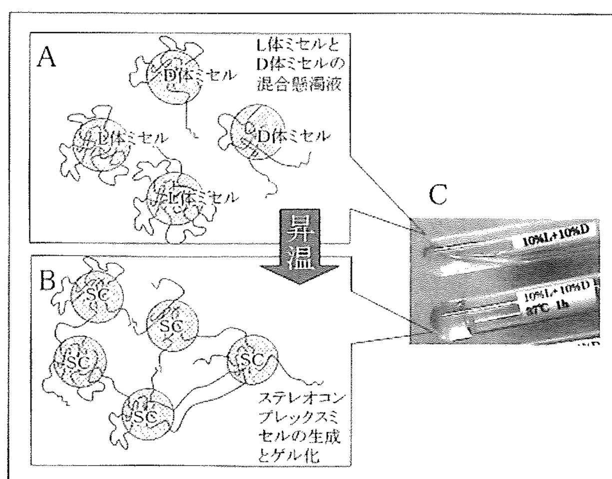


図3　L体ミセル／D体ミセル混合溶液の温度応答性ゲル化

そのゲル化メカニズムを**図3**に示した。ポリエチレングリコール-ポリ乳酸-ポリエチレングリコールという構造のトリブロック共重合体は，水溶液中でナノミセルを形成する。そこで，ポリ-L-乳酸からなるミセル(L体ミセル)と，ポリ-D-乳酸からなるミセル(D体ミセル)の分散液を混合する(図3A)。この溶液を加熱すると，隣接するL体ミセルとD体ミセルが融合し，ステレオコンプレックスミセル(SCミセル)が形成する。すなわち，L体結晶とD体結晶が融合し，新たに融点が約50℃も高い安定なSC結晶を生成する。このことにより，架橋剤や感温性分子を使うことなく，温度応答性のゲル化が可能となった。実際に，共重合組成などを調節して37℃でゲル化することに成功したインジェクタブルスキャホールドの写真を図3Cに示した。X線散乱測定により，温度上昇とともにステレオコンプレックス結晶が成長することがそのメカニズムであることも証明された。得られたゲルの含水率は90%以上であり，その物質透過性に優れた。さらに，緑色蛍光タンパク(GFP)組換え細胞の移植実験により，細胞生存率を下げることなく，また，ゲル内での長期細胞生存を可能にする物質透過性を有し，対象部位に細胞を注入できる材料であることが，明らかとなっている。

(2)幹細胞トラッキング

もう1つの課題は，送達した細胞成分の*in vivo*での動態が不明なことである。特に自己細胞移植では移植細胞を区別して観察することは容易ではなく，移植による治癒メカニズムも解明されていない。1つの手法として，GFP陽性細胞をGFP陰性マウスに移植して*in vivo*蛍光イメージング装置で追跡する手法があるが，蛍光の特性により，ラット程度の小動物が限界である。最近，われわれは移植した細胞をMRIで低侵襲的に追跡するための，新たな細胞標識用MRI造影剤の開発に成功した。**図4**に本システムの概要を示した。ガドリニウム錯体分子(丸印)の細胞膜透過性を抑制し，かつ，細胞に対する毒性を軽減させるために高分子キャリアーを用い，微弱な電気的ショックを細胞に加える手法により，あらゆる細胞に対して容易に送達することに成功した[18]。NIH3T3細胞，およびラット間葉系幹細胞，ラットEPCを標識したところ，その細胞増殖性は非標識細胞と同程度であり，その分化能力も維持されており，さらに，30日間にわたる観察でも造影剤の漏出は認められなかった。ラット下肢虚血モデルラットに移植したEPCが虚血部位へとマイグレートしている挙動がMR撮像によりとらえられている。また，細胞移植による肉腫生成も低侵襲的に追跡できる可能性が示唆されており，現在，詳細な細胞トラッキング解析を進めている。本造影剤で移植細胞数や移植回数を最低限に抑えることで，リスク回避と治療効果の最適化が可能になればと期待している。

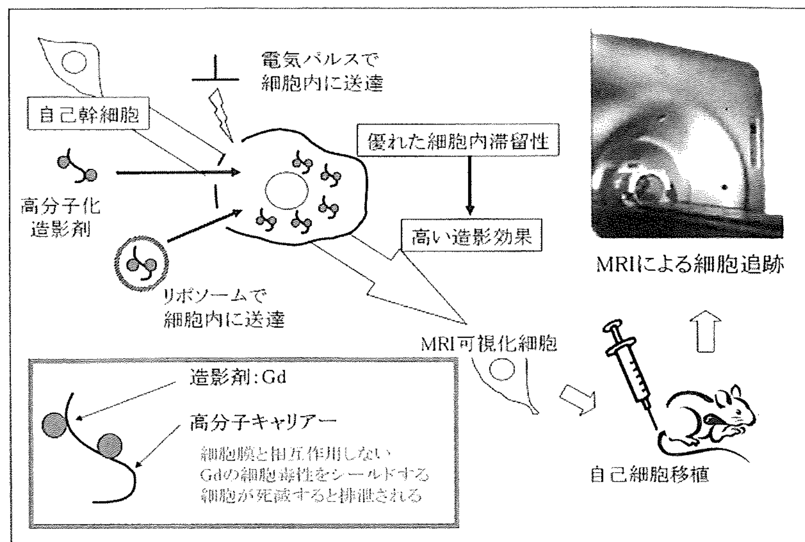


図4　Gd系水溶性MR造影剤による幹細胞トラッキング

■引用文献

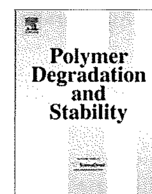
1) 医療機器・用品年間2008年版(株式会社アールアンドディ)
2) J. Aoki, P.W. Serruys, H. van Beusekom, A.T.L. Ong, E.P. McFadden, G.S. Willem, J. van der Giessen, E. Regar, P.J. de Feyter, H.R. Davis, S. Rowland, M.J.B. Kutryk：J Am. College Cardiol. **45**(10), (2005)
3) T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. Van Der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner：Science, **275**, 964-967(1997)
4) R. Blindt, F. Vogt, I. Astafieva, C. Fach, M. Hristov, N. Krott, B. Seitz, A. Kapurniotu, C. Kwok, M. Dewor, A-K. Bosserhoff, J. Bernhagen, P. Hanrath, R. Hoffmann, C. Weber：J Am College Cardiol., **47**(9)(2006)
5) G. Matsumura, S. Miyagawa-Tomita, T. Shin' oka, Y. Ikada, H. Kurosawa：Circulation, **108**(14), 1729-34(2003)
6) 再生医療用支持体，血管再生用支持体，神経再生用支持体及び治療方法, 特願2003-412423
7) R. Langer, J.P. Vacanti, Science, **260**, 920-926(1993)
8) http://www.jpte.co.jp/Professional/JACE/index.html
9) M.J. Evans, M.H. Kaufman：Nature, **292**, 154-156(1981)
10) G.R. Martin：PNAS, **78**, 7634-7638(1981)
11) J.A. Thomson：Science, **282**, 1145-1147(1998)
12) I.A. Memon, Y. Sawa, N. Fukushima, G. Matsumiya, S. Miyagawa, S. Taketani, S. K. Sakakida, H. Kondoh, A. N. Aleshin, T. Shimizu, T. Okano, H. Matsuda：J Thor. Cardiovasc. Surg., **130**(5), 1333-1341(2005)
13) Y. Miyahara, N. Nagaya, M. Kataoka, B. Yanagawa, K. Tanaka, H. Hao, K. Ishino, H. Ishida, T. Shimizu, K. Kangawa, S. Sano, T. Okano, S. Kitamura, H. Mori：Nature Medicine, **12**, 459-465(2006)
14) L. Mazzini, I. Ferrero, V. Luparello, D. Rustichelli, M. Gunetti, K. Mareschi, L. Testa, A. Stecco, R. Tarletti, M. Miglioretti, E. Fava, N. Nasuelli, C. Cisari, M. Massara, R. Vercelli, G.D. Oggioni, A. Carriero, R. Cantello, F. Monaco, F. Fagioli：Exper. Neurol. on line(2009)
15) S. Akita, K. Akino, T. Imaizumia, A. Hirano, Burns, **31**, 855-858(2005)
16) Babensee, J.E.a, McIntire, L.V.b , Mikos, A.G.b　Pharmaceutical Research：**17**, 497-504(2000)
17) T. Fujiwara, T. Mukose, T. Yamaoka, H. Yamane, S. Sakurai, Y. Kimura：Macromol. Biosci., **1**, 204-208(2001)
18) Y. Tachibana, J. Ennmi, H. Iida, T. Yamaoka, Abstract for SFB 2007 Annual meeting, **94**(2007)

Polymer Degradation and Stability 94 (2009) 1349–1353

Contents lists available at ScienceDirect

Polymer Degradation and Stability

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



Self-assemblies of enzymatically degradable amphiphilic oligopeptides as nonviral gene carrier

Tomoko Hashimoto [a,b], Reiko Iwase [b,c], Akira Murakami [b], Tetsuji Yamaoka [a,b,*]

[a] Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
[b] Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan
[c] Department of Biosciences, Teikyo University of Science and Technology, 2525 Yatsusawa, Uenohara, Yamanashi 409-0193, Japan

ARTICLE INFO

Article history:
Received 25 April 2009
Received in revised form
25 May 2009
Accepted 28 May 2009
Available online 6 June 2009

Keywords:
Polymeric gene carrier
Self-assembly
Stability
Oligopeptide
Molecular weight

ABSTRACT

Novel biodegradable oligopeptide-type gene carriers composed of cationic residues (KRRRKRKRRRKRKRRC) and oligo leucine segments were developed. The amphiphilic carrier was found to form micelle-like assemblies in aqueous solutions, when the oligo leucine is 12 amino acids length (Pep-L12). NMR, CMC, and GPC analysis revealed their hydrophobic/cationic core/shell morphology. Hydrophobic interaction between leucines is thought to be the major driving force behind formations of assemblies. The transient expression of luciferase introduced to COS-1 cells using Pep-L12 below the CMC is as low as that by the control cationic peptides without leucine residue (Pep-L0), while improved transgene expression was observed in the case of Pep-L12 above CMC. The self-assembly raised the apparent molecular weight and gene transfection ability without loosening their low cytotoxicity. These results indicate that the amphiphilic oligopeptides are very promising materials as highly efficient and less toxic gene carriers.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Polymeric gene carriers are now extensively studied due to their high abilities to deliver and protect pDNA, but the polyplexes formed between the carriers and pDNAs are sometimes too highly compacted to be recognized by transcription factors in nucleus. Recently, the destabilization of the polyplexes by conjugating hydrophilic or hydrophobic segments to polymeric carriers has been reported [1–3]. However, excess modification of side chains results in the low resistance to DNase at the same time.

Effect of the carrier molecular weights (Mws) has been also being studied [4–8]. Recently, high potential of low Mw polymeric carriers was attracting great attention. Kunath et al. reported that low Mw PEI (5 kDa) was much less toxic than high Mw PEI (48 kDa), and reporter gene expression of 5 kDa PEI was 3.7-fold higher than 48 kDa PEI in various cell lines [6]. Breuning et al. compared PEIs with the Mw of 1–9 kDa and showed that the highest reporter gene expression was obtained at 5.6 kDa, with low cytotoxicity. Schaffer et al. reported higher gene expression for low Mw PLL (19 and 36 residues) than high Mw PLL (180 residues) because of effective *in vitro* transcription and easy pDNA release [4]. Taken together, low cytotoxicity and high DNA releasing ability of low Mw carries were important key features for the high potential gene carriers. On the other hand, low Mw carriers are pointed out to reduce cellular uptake [9] and decrease stability of polyplexes at the same time. Thus, a new type "low Mw carriers", which have low cytotoxicity, high cellular uptake, and adequate polyplex stability, would be more useful gene carriers.

In the present study, oligopeptide-type carriers were selected in order to reduce cytotoxicity and to induce the intracellularly digestible feature. Since the chemical chain elongation of cationic oligopeptide would increase the cytotoxicity, we tried to raise the apparent Mw of oligopeptide-type carriers by their self-assembly. Amphiphilic oligopeptides having cationic and hydrophobic sequences were then designed. Hydrophobic interactions between oligo leucine sequences make carriers form assemblies and increase the apparent Mw. Cationic sequences for interacting with pDNA include cleavable sequences (Arg-X-Lys/Arg-Arg (R-X-K/R-R)) by intracellular proprotein convertase, furin [10,11]. We have previously found that carriers including this cleavable sequences are enough cationic to form polyplexes with pDNA, and these polyplexes became destabilized if carriers were cleaved by furin [12]. Increased apparent Mw is expected to increase cellular uptake of the polyplex and the enhanced stability can be destabilized by furin cleavage resulting in the pDNA release in intracellular environments.

* Corresponding author at: Department of Biomedical Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012x2637; fax: +81 6 6835 5476.
E-mail address: yamtet@ri.ncvc.go.jp (T. Yamaoka).

0141-3910/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymdegradstab.2009.05.021

2. Experimental

2.1. Amphiphilic oligopeptides

We synthesized four oligopeptides by Fmoc-based solid phase method using 9050 plus PepSynthesizer (Applied Biosystems, CA, USA) and purified in the usual way. They are composed of cationic KRRRKRKRRRKRKRRC and hydrophobic oligo leucine segment with different lengths.

Oligopeptide solutions were analyzed by GPC (Shimadzu Corporation, Kyoto, Japan) which fitted with a combination of two columns of TSK gel G6000PWXL (21.5 mm I.D. × 300 mm length, Tosoh Corporation, Tokyo, Japan) and TSK gel G3000PWXL, RID-10A Refractive index detector, and SPD-M10A UV–VIS detector. Elution was carried out with 1/15 M phosphate buffer (pH 7.5) at 0.3 mL/min.

2.2. Critical micelle concentration (CMC) measurements

CMCs of oligopeptides in aqueous solution were measured on a RF5300PC (Shimadzu Corporation, Kyoto, Japan) using pyrene (Nacalai Tesque, Inc., Kyoto, Japan) as a hydrophobic region probe [13]. Five μL of pyrene solution in acetone at a concentration of 6×10^{-5} M was transferred into a vial and evaporated. Five hundred μL of oligopeptide solutions which ranging from 5.0×10^{-4}–1.5 g/L were added dropwise to make the pyrene concentration of 6.0×10^{-7} M, incubated at 65 °C for 3 h, and cooled down to the room temperature. Pyrene excitation spectra were measured with the slit widths of 5 and 1.5 nm for excitation and emission at an emission wavelength of 380 nm.

2.3. Polyplex formation with pDNA

pCMV-Luc and pT7-Luc (Promega corporation, WI, USA) were amplified to sufficient quantities by standard molecular biology techniques, and purified with a QIAGEN-tip 500 (QIAGEN K.K., Tokyo, Japan). Oligopeptide solutions were mixed with pDNA solutions at a given charge ratio which is the ratio of the number of cationic groups of oligopeptide to that of anionic group of pDNA (*C/A* ratio). The solutions were incubated for 30 min at 37 °C to allow the polyplex formation and analyzed on 0.8 wt% agarose gel in Tris–borate EDTA buffer at 100V for 30 min. pDNA was visualized by staining with 0.5 μg/mL ethidium bromide (EtBr, Sigma chemicals, St Louis, MO, USA).

2.4. *In vitro* transfection

COS-1 cells were grown in DMEM (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Sigma chemicals, USA) at 37 °C under a 5% CO_2 atmosphere. COS-1 cells were seeded in 96 well culture plates at a density of 1×10^4 in 100 μL DMEM containing 10% FBS per well. After 24 h incubation, cells were washed with PBS, and 40 μL DMEM was added. Ten μL of polyplex solutions containing 100 ng pCMV-Luc at the concentration above or below CMC of Pep-L12 were poured gently to the wells. Fifty μL of 200 μM chloroquine solution was added (final concentration is 100 μM) and incubated for 5 h. Cells were washed with PBS and cultured for 43 h with DMEM containing 10% FBS at 37 °C in a 5% humidified CO_2 environment. The cells were washed with PBS, treated with the lysis buffer containing 1% Triton-X100, and incubated for 30 min at 37 °C. Cell lysate was diluted into luciferase assay solution containing 470 μM luciferin. The relative light units (RLU) of expressed luciferase were measured using ATP-300 Lumicounter (Advantec Toyo Kaisya, Ltd., Tokyo, Japan). Luciferase solutions at a known concentration were used for calibration. The protein concentration was determined by DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. The obtained luciferase expression (ng luciferase) was divided by total protein content of cell lysates and expressed as ng luciferase/mg protein.

Table 1
Sequences of amphiphilic oligopeptides.

Oligopeptide	Sequences and cleavage sites	Amino acid composition (K/R/L/C)
Pep-L0	H_2N-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/0/1
Pep-L4	H_2N-$(L)_4$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/4/1
Pep-L8	H_2N-$(L)_8$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/8/1
Pep-L12	H_2N-$(L)_{12}$-K-R-R-R-K-R*-K-R-R*-R-K-R*-K-R-R*-C-$CONH_2$	10/5/12/1

* represents the cleavage site of furin.

2.5. Cell-free assay system for luciferase expression

Fifteen μL of polyplexes ($C/A = 10$) were mixed with 12.8 μL of rabbit reticulocyte lysate mixtures (T_NT Coupled Reticulocyte Lysate Systems; Promega, WI, USA) and incubated with shake at rate of 300 rpm/min for 90 min at 30 °C. After transcription/translation assay according to the manufacture's protocol, luciferase activities were measured by the same method described in the above section.

3. Results and discussion

3.1. Self-assembly of amphiphilic carriers

Sequences and abbreviation of synthesized amphiphilic oligopeptides were shown in Table 1. GPC chart for each amphiphilic oligopeptide in phosphate buffer is shown in Fig. 1. Only Pep-L12 exhibited two peaks, while the other oligopeptides showed peak which is at the similar elution time to the second peak of Pep-L12. The first peak of Pep-L12 is considered to be attributed to the self-assembly of the Pep-L12 with the apparent higher Mw and the second peak corresponds to the unimer as low Mw as the other oligopeptides, Pep-L0, Pep-L4, and Pep-L8. These results indicated that only Pep-L12 forms micelle-like assemblies in aqueous solution.

Micelle-like assemblies can be confirmed by comparing ^1H NMR spectra in good solvents and water [14,15]. Protons in the core structure composed of the insoluble fractions do not provide sufficient NMR signals. Thus, self-assembly of oligopeptides was analyzed in DMSO and water. Leucine contents (X_{Leu}) in water and in DMSO were measured using the signal intensity at 0.8 ppm (CH_3 in leucine) and at 1.6 ppm (β-CH_2 and γ-CH in leucine, β, γ, and δ-CH_2 in lysine, β and γ-CH_2 in arginine, and SH in cysteine). The

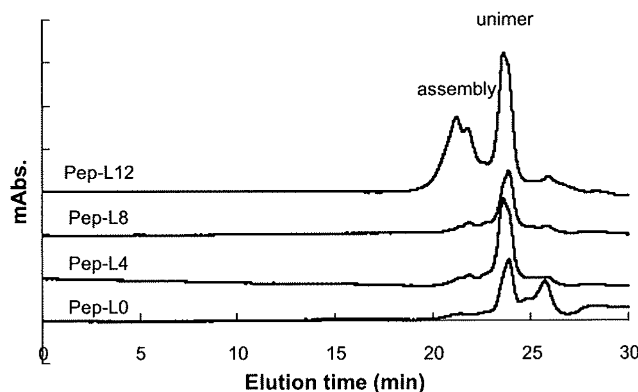


Fig. 1. GPC charts of Pep-LX ($X = 0, 4, 8$ and 12).