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Original Article

Defining Patients at Extremely High Risk for Coronary Artery Disease in Heterozygous Familial Hypercholesterolemia

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Aim: Heterozygous patients with familial hypercholesterolemia (FH) are known to be associated with a high risk of coronary artery disease (CAD), which is a major determinant of their clinical outcome. The prognosis of heterozygous FH patients substantially varies, being dependent on the level of their CAD risk, and their therapeutic regimen should be individualized. We assessed critical levels of LDL-cholesterol (LDL-C) and Achilles tendon thickness (ATT) to identify heterozygous FH patients at "very high" risk for CAD.
Methods: One hundred and nine heterozygous FH patients who had no history of CAD and had had their plasma lipid profile and ATT assessed before treatment were followed up until their first CAD event or 31 December 2010. Multivariable logistic regression models were used to analyze the correlation of LDL-C and/or ATT levels with the risk of developing CAD.
Results: During the follow-up period, 21 of the 109 patients had a CAD event, diagnosed by coronary angiogram. Individuals in the highest tertile of LDL-C had a CAD risk 8.29-fold higher than those in the lowest tertile. Individuals in the highest tertile of the ATT group had a 7.82-fold higher CAD risk than those in the lowest tertile. Those who had either LDL-C ≥260 mg/dL or ATT ≥14.5 had a 23.94-fold higher CAD risk than those with LDL-C <260 mg/dL and ATT <14.5 mm.
Conclusions: In heterozygous FH patients, LDL-C 260 mg/dL or higher and/or ATT 14.5 mm or thicker are useful markers for extracting patients at "very high" risk for CAD.

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Key words; Familial hypercholesterolemia, LDL cholesterol, Coronary artery disease, Coronary risk, Achilles tendon thickness

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by hypercholesterolemia, skin and tendon xanthomas and a high risk of coronary artery disease (CAD) due to premature atherosclerosis[1]. FH has the highest prevalence in genetic metabolic diseases, showing one per 300 to 500 heterozygous patients in the general population[1, 2]. High low-density lipoprotein cholesterol (LDL-C) is the first symptom, appearing in heterozygous FH even from birth, and xanthomas in the Achilles tendon usually appear during or after the late 10s and are found in half of all patients by the age of 30[1]. Coronary artery disease (CAD), which determines the prognosis of FH patients, appears during or after the third decade of life in men and the fifth decade in women[3-5].

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CAD mortality in heterozygous FH is several times higher than that in the general population[1, 6-8]; therefore, it is very important to prevent CAD in heterozygous FH patients. The prognosis of heterozygous patients of FH varies substantially, such that some develop CAD at their 20s while others may not develop CAD until their seventh decade; therefore, the therapeutic regimen should be individualized according to the patients' risk of CAD.

Various risk factors for CAD have been identified in heterozygous patients with FH, such as age, sex, LDL-C, triglyceride (TG), HDL-C, Achilles tendon thickness (ATT), smoking, a family history of CAD, hypertension, diabetes mellitus, Lp(a), homocysteine and so on[3, 9-12]. Among these parameters, LDL-C and ATT are simple, specific and non-invasive to measure, and can easily be used by primary care physicians to evaluate the CAD risk. We therefore estimated the CAD risks in accordance with LDL-C and ATT in heterozygous FH patients in order to identify those at extremely high risk.

Methods

Subjects

Of the patients referred to the lipid clinic at the National Cerebral and Cardiovascular Center (NCVC) from 1977 to 2007, 329 consecutive patients diagnosed as FH heterozygotes using previously described criteria[6] were subjected to this study. After diagnosis, the FH patients had medical checks according to the standard procedure for treating heterozygous FH in NCVC. The patients were subjected to a treadmill test for CAD screening just after their first visit to our clinic. Those who had a positive result on the treadmill test were subjected to a coronary angiogram (CAG), and diagnosed with CAD with 75% or more stenosis. Those who had a negative result on the treadmill or no significant stenosis by CAG were included in this study. Among the 329 FH patients, 229 were excluded: 53 had a past history of CAD, 160 had not had LDL-C measured before treatment, 76 had not had ATT thickness measured and 3 had TG more than 4.5 mmol/L, so 109 were followed up until their first CAD event or 31 December 2010. After the first visit to our clinic, dietary and drug treatment, including statins, was immediately started and continued.

During the course, those who had chest pain or a positive result on the treadmill test performed biennially were subjected to CAG, and diagnosed with CAD with 75% or more stenosis. Medical records of the patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2).

Clinical and Laboratory Characteristics

Serum lipid and lipoprotein levels were measured at the time of initial diagnosis, before any lipid-lowering treatment. TC, TG and HDL-C levels were measured enzymatically with a commercial kit (Daiichi Pure Chemicals Co., Tokyo, Japan) using an automated analyzer (Hitachi model 704; Hitachi, Tokyo, Japan) in the clinical laboratory of the National Cerebral and Cardiovascular Center (NCVC). LDL-C was calculated by the Friedewald formula. ATT was measured by X-ray according to the method previously described[13]. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or blood pressure ≥ 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)[14]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japanese Diabetes Society[15]. A family history of CAD was defined as positive by having within 2nd degree family members with CAD on the standardized questionnaire. Smoking was defined as positive by having a smoking habit at the first visit to NCVC on the patient report.

Statistical Analyses

Continuous variables are presented as the means ± SDs. Categorical data are presented as numbers and percentages. Unpaired Student's t-test and one-way analysis of variance (ANOVA) were used to assess differences between groups in continuous variables. Differences in categorical variables were assessed by the χ^2 test.

Multivariable logistic regression analysis after adjusting for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL) were used to analyze correlations of LDL-C levels or ATT levels and the development of CAD. LDL-C levels were categorized into tertiles: (1) LDL-C <206 mg/dL, (2) LDL ≥206 and <260 mg/dL, (3) LDL-C ≥260 mg/dL. ATT levels were also categorized into tertiles: (1) ATT <9.0 mm, (2) ATT ≥9.0 mm and <14.5 mm, (3) ATT ≥14.5 mm. All the confidence intervals were estimated at the 95% level and significance was set at $p<0.05$. All data were analyzed with the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

Table 1. Clinical characteristics of 109 patients with heterozygous FH classified with or without CAD

	Total $n=109$	CAD(−) $n=88$	CAD(+) $n=21$	p value
Age (years)	41.9 ± 16.2	39.7 ± 16.7	50.9 ± 10.5	<0.01
Sex (male), n (%)	43 (39.4%)	30 (34.1%)	12 (57.1%)	0.052
Achilles tendon thickness (mm)	12.6 ± 5.4	11.5 ± 4.5	17.4 ± 6.3	<0.0001
Skin xanthomas, n (%)	25 (22.9%)	16 (18.2%)	9 (42.9%)	0.052
Arcus cornea, n (%)	45 (41.3%)	27 (30.7%)	16 (76.2%)	0.001
Total cholesterol (mg/dL)	321 ± 68	309 ± 56	368 ± 92	<0.001
Triglyceride (mg/dL)	139 ± 82	134 ± 85	156 ± 65	0.272
HDL-C (mg/dL)	51 ± 15	51 ± 15	50 ± 15	0.747
LDL-C (mg/dL)	242 ± 70	232 ± 59	287 ± 92	0.001
Smoking (past or current), n (%)	42 (38.5%)	28 (31.8%)	14 (66.6%)	0.003
Hypertension, n (%)	19 (17.4%)	10 (11.4%)	9 (42.9%)	0.003
Diabetes mellitus, n (%)	9 (8.2%)	5 (5.7%)	4 (19.0%)	0.186
Family history of CAD, n (%)	47 (43.1%)	37 (42.0%)	10 (47.6%)	0.411

Table 2. Clinical characteristics at first visit in heterozygous patients of FH classified by LDL-C Levels (Mean ± SD)

LDL-C (mg/dL) categories	LDL-C <206 $n=36$	206≦LDL-C<260 $n=36$	LDL-C ≥260 $n=37$	p value
Age (years)	43.7 ± 15.6	42.0 ± 17.5	40.0 ± 15.8	0.645
Sex (male), n (%)	14 (38.9%)	13 (36.1%)	15 (40.5%)	0.928
Body mass index (kg/m^2)	22.2 ± 3.3	22.5 ± 3.2	22.8 ± 6.8	0.880
Total cholesterol (mg/dL)	258 ± 28	308 ± 20	394 ± 55	<0.001
Triglyceride (mg/dL)	149 ± 102	134 ± 67	134 ± 74	0.672
HDL-C (mg/dL)	54 ± 16	51 ± 15	47 ± 14	0.100
Smoking (past or current), n (%)	15 (41.7%)	10 (27.8%)	15 (40.5%)	0.385
Hypertension, n (%)	5 (13.9%)	6 (16.7%)	3 (8.1%)	0.660
Diabetes mellitus, n (%)	2 (5.6%)	3 (8.3%)	2 (5.4%)	0.831
Family history of CAD, n (%)	17 (47.2%)	14 (38.9%)	16 (43.2%)	0.775
Achilles tendon thickness (mm)	10.7 ± 4.2	12.5 ± 5.5	14.6 ± 5.8	0.282
CAD, n (%)	5 (13.9%)	3 (8.3%)	13 (35.1%)	0.02

Results

Characteristics of the Patients Subjected to Analysis of the Correlations of LDL-C and CAD

Among 109 patients, 21 (19.3%) developed CAD during the subsequent period. There was a significantly higher prevalence of hypertension, skin xanthomas, arcus cornea and smoking in the CAD (+) group. Mean age, ATT, TC and LDL-C were significantly higher in the CAD (+) group than in the CAD (−) group (**Table 1**).

LDL-C Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their LDL-C levels are shown in **Table 2**. They clearly show that parameters other than TC levels were not significantly different in each tertile. Higher LDL-C was associated with higher TC and the incidence of CAD.

To examine the influence of conventional coronary risk factors, logistic regression analyses for CAD were performed. The multivariable adjusted odds ratios (ORs) for CAD are shown in **Table 3**. Individuals in the highest tertile (LDL-C ≥260 mg/dL) had a 8.29-fold increased risk of CAD incidence compared with those in the lowest tertile (LDL-C <206 mg/dL) (adjusted odds ratio (OR) 8.29, 95 % CI 1.33-51.47, $p=0.023$). No significant increase in the odds of future CAD in the second tertile (206≦LDL-C<260 mg/dL) (adjusted OR 0.42, 95%CI 0.05-3.26, $p=0.409$).

Table 3. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to LDL-C

LCL-C categories	n	Odds Ratio	95% CI	*p* value
LDL-C <206 mg/dL	36	1.0 (referent)	–	–
206≦LDL-C<260 mg/dL	36	0.42	0.05-3.26	0.409
LDL-C ≥260 mg/dL	37	8.29	1.33-51.47	0.023

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL).

Table 4. Clinical characteristics at first visit in heterozygous patients of FH classified by ATT levels (mean ± SD)

ATT (mm) categories	ATT <9 *n*=36	9≦ATT<14.5 *n*=37	ATT ≥14.5 *n*=36	*p* value
Age (years)	39.7±18.3	39.4±16.4	45.2±13.5	0.177
Sex (male), n (%)	11 (30.6%)	13 (35.1%)	18 (50.0%)	0.207
BMI (kg/m^2)	22.3±2.8	21.7±2.8	23.1±2.7	0.883
Total cholesterol (mg/dL)	293±42	319±66	350±79	0.002
Triglycerides (mg/dL)	140±106	134±69	142±67	0.505
HDL-C (mg/dL)	57±14	47±14	48±15	0.916
LDL-C (mg/dL)	208±44	245±67	274±78	0.003
Smoking habit, n (%)	9 (25.0%)	13 (35.1%)	14 (38.9%)	0.001
Hypertension, n (%)	4 (11.1%)	2 (5.4%)	8 (22.2%)	0.094
Diabetes mellitus, n (%)	1 (2.8%)	1 (2.7%)	5 (13.9%)	0.125
Family history of CAD, n (%)	16 (44.4%)	17 (46.0%)	14 (38.9%)	0.815
CAD, n (%)	2 (5.6%)	4 (10.8%)	15 (41.7%)	<0.001

ATT Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their ATT levels are shown in **Table 4**. Higher ATT levels were associated with higher TC and LDL-C levels, smoking and the incidence of CAD.

The multivariable adjusted OR for CAD is shown in **Table 5**. Individuals in the highest tertile group of ATT ≥14.5 mm had a 7.82-fold increased risk of CAD compared with those in the ATT <9.0 mm group (95%CI 1.28-47.7, p=0.001). No significant increase in the odds of future CAD in the group with 9≦ATT<14.5 mm (adjusted OR 1.42, 95%CI 0.18-11.14, p=0.740).

LDL-C and/or ATT Levels and Development of CAD

To estimate the future risk for CAD using the combination of LDL-C and ATT thickness, the patients were divided into 3 groups, (1) LDL-C <260 mg/dL and ATT <14.5 mm, (2) LDL-C <260 and ATT ≥14.5, or LDL-C ≥260 and ATT <14.5, (3) LDL-C ≥260 and ATT ≥14.5. OR for CAD was calculated for these groups and shown in **Table 6**. Those who had both LDL-C ≥260 and ATT ≥14.5 had a 20.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT <14.5 (95%CI 2.91-145.89). Those with either LDL-C ≥260 or ATT ≥14.5 had a 23.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT < 14.5 (95%CI 3.11-184.16).

Discussion

As the prognosis of heterozygous FH patients varies substantially, the therapeutic regimen should be determined according to the CAD risk of individual patients. High levels of LDL-C and ATT are clinical signs already found at a young age and can be measured easily and non-invasively by family physicians in primary care, so they can be good markers for estimating the future CAD risk of FH. In the present study, we demonstrated the critical levels of LDL-C and ATT for estimation of the CAD risk in Japanese heterozygous patients with FH.

Several studies on the Japanese population have indicated that the serum cholesterol level is correlated significantly with the risk of CAD[16, 17]. The increased CAD incidence seems exponential with the serum cholesterol level in the general population, and it can be

Table 5. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to ATT levels

ATT (mm) categories	n	Odds Ratio	95% CI	*p* value
ATT <9 mm	36	1.0 (referent)	-	-
9≦ATT<14.5 mm	37	1.42	0.18-11.14	0.740
ATT ≥ 14.5 mm	36	7.82	1.28-47.7	0.001

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (<40 mg/dL).

Table 6. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to both ATT and LDL-C levels

LDL-C and ATT categories	n	Odds Ratio	95% CI	*p* value
LDL-C <260, ATT <14.5 mm	54	1.0 (referent)	-	-
LDL-C <260, ATT ≥ 14.5 mm or LDL-C ≥ 260, ATT <14.5 mm	37	23.94	3.11-184.16	0.002
LDL-C ≥ 260, ATT ≥ 14.5 mm	18	20.62	2.91-145.89	0.002

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (<40 mg/dL).

considered low until it hits a certain "threshold". The findings of the relationship with LDL-C levels and the onset of CAD in FH patients seem to show a "right shift" of this profile as LDL-C 2-fold higher and CAD incidence more than 10-fold. Previous studies have reported that higher LDL-C is related with the higher risk factors for the development of CAD even in heterozygous FH patients[18, 19], whereas other factors, such as age, gender, hypertension, smoking, or other lipid abnormalities, such as low HDL-C and high TG reportedly contribute to the increased risk[3, 12, 18-22]. Bujo *et al.* reported that male gender, age over 50, smoking, hypertension, diabetes mellitus, TG >150 mg/dL and HDL-C <40 mg/dL were risk factors for CAD in FH by multicenter, cross-sectional analysis[3].

As we reported in a previous paper, drug treatment including statins may influence the outcome of CAD[5]. The name and dose of drugs prescribed to the patients during the course are listed in **Table 7**. Because all FH patients had had intensive drug therapy to prevent the development of atherosclerosis, no comparison could be made with those without drug therapy. It was also impossible to analyze the difference in drugs statistically because there were so many patterns of prescription and most patients changed the type and dose of drugs several times during the course.

LDL-C levels under drug treatment may also affect the outcome. The mean LDL-C under drug treatment did not increase the odds ratio for CAD (odds ratio: 0.983, 95%CI: 0.97-1.00); however the relationship between mean LDL-C in the pre-treatment period and CAD risk remained due to pre-exposure to high LDL-C before treatment, although the absolute risk of CAD might be decreased at any LDL-C level by intensive drug treatment during the course.

Table 7. Lipid-lowering drugs in heterozygous FH patients during the course

	Dose/day
cholestyramine	4-12 g
colestimide	0.5-3 g
probucol	250-1,000 mg
pravastatin	10-30 mg
simvastatin	5-20 mg
fluvastatin	20-60 mg
atorvastatin	5-40 mg
pitavastatin	1-4 mg
rosuvastatin	2.5-20 mg
fenofibrate	100-200 mg
bezafibrate	100-400 mg
ezetimibe	5-10 mg

Civerira *et al.* reported that heterozygous FH with tendon xanthomas has a 3.1-fold increased risk of premature CAD compared with those without it[23]. The Achilles tendon was reported to be thicker in FH patients with CAD than in those without CAD in both sexes[12]. Persistent high LDL-C causes cholesterol depositions in the tendons and results in tendon xanthomas[1]. Achilles tendon xanthomas have been used

as one of the criteria for clinical diagnosis of FH because of their high sensitivity and specificity[1, 24]. A strongly positive correlation was observed between ATT and cholesterol-year scores in FH patients[25, 26], suggesting that ATT reflects both the severity and duration of hypercholesterolemia. ATT is an important factor that can be measured quantitatively as the deposition of cholesterol in the tissue. The present study showed that ATT is a good marker for evaluating the risk for CAD, indicating that there is a strong correlation between the deposition of cholesterol in extravascular tissue and the stenosis of coronary arteries. ATT should be used not only as a diagnostic parameter for FH but also, and more importantly, as a prognostic factor that indicates the need for a more aggressive approach for patients at high risk.

In conclusion, LDL-C $\geq$260 mg/dL and ATT $\geqq$14.5 mm or thicker are useful criteria for identifying patients at "very high" risk of CAD in Japanese heterozygous FH. Patients with either of these risk factors require more intensive cholesterol-lowering therapy and a more careful medical check-up for CAD.

Acknowledgments

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Special Report

Background to Discuss Guidelines for Control of Plasma HDL-Cholesterol in Japan*

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A decrease in high density lipoprotein-cholesterol (HDL-C) is a strong risk factor for atherosclerotic disorders in Japan, probably more important than an increase in low density lipoprotein-cholesterol (LDL-C). While there are rational grounds for the argument that elevation of HDL-C leads to decreased risk, there has as yet been no direct evidence of such an effect. If elevation of HDL-C decreases the risk, this effect is expected throughout the normal range of HDL-C or perhaps even higher than that. Simulation based on epidemiological data indicated that it may eventually reduce the incidence of ischemic heart disease by 60-70% in Japan. In the risk management guideline, "low" HDL-C is presently defined as 40 mg/dL or below. While there is no evidence that strongly urges a change in this definition, the results of epidemiological studies support "The higher the HDL-C level, the lower the risk," even in the "normal range". Elevation of the HDL-C level may reduce the risk, probably at least up to 70 mg/dL; however, there are no supportive data for this effect still being obtained over 80 mg/dL. Patients with homozygous CETP deficiency should be followed-up while controlling other risk factors, so as not to dismiss the possibility of a risk increase with an extremely elevated HDL-C level.

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Clinical Relevance of HDL-C Management

Numbers of epidemiological studies have established that the risk of coronary artery disease increases as plasma HDL-C decreases, and decreases as it increases. In addition, many experimental approaches

have demonstrated that cholesterol is extracted by HDL particles in the culture medium from cultured cells, including macrophages overloaded with cholesterol.

From these two lines of evidence, HDL is believed to be a "preventive factor" against atherosclerosis. This view is strongly associated with the hypothesis that HDL plays a central role in the recovery of cholesterol molecules from tissues and organs, which cannot be catabolized in peripheral cells, and in their transport to the liver for conversion to bile acids. From the viewpoint of public health, many research results suggest that a decrease in HDL-C contributes more than an increase in LDL-C to the development of ischemic heart disease in Japan. In studies conducted at Nagoya City University, for example, narrowing of the coronary artery was more closely related to triglycerides (TG) and HDL-C than to total cholesterol (TC) or LDL-C[1, 2], and this tendency is commonly observed in many other reports. HDL-C is thus suggested to be a strong determinant of atherosclerosis in Japan and perhaps a more important risk factor than LDL-C from a public health point of view.

HDL is smaller (12 nm or less in diameter) than other lipoproteins, abundant in protein and does not contain much TG, so it has a greater hydrated density than other lipoproteins (d=1.063-1.21). Similarly to other plasma lipoproteins, however, HDL functions to transport cholesterol among cells or organs using the flow of blood or extracellular fluid. Cholesterol, an essential molecule for the life of animals, requires a number of steps and plenty of energy for synthesis, and its dietary intake is not always guaranteed; therefore, the animal body has developed systems to use cholesterol sparingly as a precious material. As a result, little cholesterol is converted to energy in its catabolism, and, with the exception of a very small amount used for the production of steroid hormones, most cholesterol is transported to the liver for conversion to bile acids and is recycled an reused in the intestine before excretion. Its steroid backbone is not degraded in the metabolism in the animal body and finally broken down by microorganisms in the environment. Therefore, cholesterol molecules must be released from most somatic cells for metabolic homeostasis, and HDL receives these cholesterol molecules for their transport. Cholesterol is converted to cholesteryl acylester (CE) as a fatty acyl chain and transferred from phosphatidylcholine to its hydroxyl group to form an ester bond, for packing cholesterol molecules into the core of HDL. CE is recovered by the liver directly from HDL by a selective uptake reaction, or as LDL particles after being transferred to apolipoprotein B-containing lipoproteins by CE transfer protein (CETP). As a result of these activities, HDL is considered to exert a preventive effect against atherosclerosis as it interferes with the excessive accumulation of cholesterol in cells from LDL, etc., by extracting it.

No drug has been marketed yet to independently increases HDL-C; therefore, the question of whether increasing HDL-C is effective for preventing and treating atherosclerotic disorders has not been answered. However, researchers have recently directed more attention to HDL and, accordingly, more research results on HDL metabolism have recently accumulated. Much effort to develop drugs targeting HDL has been initiated. On the other hand, some existing drugs are known to increase plasma HDL-C. Drugs that reduce TG generally increase HDL-C, primarily because these drugs reverse low HDL-C induced by high TG through CETP[3]. In addition, fibrates have been suggested to directly increase HDL production[4]. Many clinical studies have also shown that statins elevate HDL-C as well as decreasing LDL-C. Concerning their mechanism, statins have recently been reported to increase HDL synthesis in the liver, unlike their effects in peripheral tissues[5]. The mechanism of the increase in HDL through exercise and alcohol intake has not been sufficiently elucidated. As mentioned below, the question of whether HDL-C increase by inhibiting CETP prevents atherogenesis has been shelved because of the failure to develop a CETP-inhibiting drug, perhaps due to a business-oriented strategy[6].

Position of HDL in Risk-Reducing Strategies

Large-scale clinical studies targeted to high LDL-C and high TG, major risk factors of atherosclerotic diseases, such as ischemic heart disease, have indicated that ischemic heart disease can be prevented by reducing LDL-C and TG and, particularly, that mortality due to the disease can be lowered by controlling the LDL-C level, with a consequent reduction in the total number of deaths in the high-risk group. In addition, based on stratified analysis of the results of many clinical trials, the conclusion has been reached that an increase in HDL-C contributes to the prevention of diseases as a "statistically independent factor". In consideration of the above-stated marked epidemiological contribution of HDL-C as a "negative risk factor" and the significant "indirect evidence" of an increase in HDL-C in the prevention of atherogenesis, the argument that a standard should be set for the control of HDL-C appears to be well grounded. However, it is also true that a consensus concerning

HDL-C management, similar to that in evidence-based quantitative guidelines for the control of LDL-C and the management and treatment of high TG, is difficult to reach at present, when no therapeutic technique specifically targeted to increase HDL-C has reached a practical level and there is no direct evidence concerning the prevention and treatment of atherosclerotic disorders using such a technique. Thus, any therapeutic guideline regarding HDL-C is merely a "proposal" based on indirect circumstantial evidence until the results of a large-scale clinical trial of a technique to specifically increase HDL-C become available.

Recently, some negative implications have been spread regarding the anti-atherosclerotic effect of an increase in HDL-C, inviting some confusion in the discussion. One is the discontinuation of a large-scale clinical study on the prevention of ischemic heart disease by increasing HDL-C, carried out to develop the CETP inhibitor torcetrapib, due to an increase in the mortality rate in the treated group[6]. Another is a large-scale epidemiological study reporting that a mutation to cause dysfunction of ABCA1, a rate-regulating protein of HDL biogenesis, is not likely to be a risk factor of ischemic heart disease[7]. The first report appears to support the contention of researchers arguing that "an increase in HDL-C by CETP inhibition has no anti-atherosclerotic effect," and allowed the generalized assertion that "the HDL-C increasing strategy is a mistake" to emerge; however, these reports do not necessarily mean the failure of CETP inhibitors themselves, and the pressor effect of a particular drug, torcetrapib, is likely to have led to such results. This incidence postponed an answer to the question of whether increasing HDL-C with a CETP inhibitor is a good idea, the most important medical issue, and markedly complicated the strategy for developing HDL-C elevating agents in general. Also, studies on ABCA1 mutation have shown that the maximum decrease in HDL is about 20%, suggesting that this does not necessarily reject the benefit of high HDL-C.

Under these circumstances, the position has not changed that an elevation of HDL-C is an important part of the anti-atherosclerotic strategy, including CETP inhibition. The above discussion may be summarized as follows: 1) a decrease in HDL-C is a strong risk factor for atherosclerotic disorders, 2) there are rational grounds for the supposition that this risk can be reduced by correcting low HDL-C (increasing HDL-C), but 3) no direct evidence has been obtained that increasing HDL-C is effective for the prevention and treatment of atherosclerotic disorders, 4) changes in HDL-C may include changes in the number and size of HDL particles, and the difference in their clinical significance may become a problem in the future.

Simulation of Atherosclerosis Prevention by Increasing HDL-C

There are qualitative scientific grounds for lowering the LDL-C level to reduce the risk of atherosclerotic disorders or, more specifically from an evidence-based viewpoint, to reduce the probability of the occurrence of ischemic heart disease; however, to prepare specific guidelines for diagnosis and treatment, quantitative criteria are considered indispensable. This is a problem with the concept in setting therapeutic goals for target groups. A quantitative profile of increases in the risk associated with elevations of the LDL-C level is necessary, and, if possible, results directly showing that the treatment reverses this curve of increasing risk must be presented. It is not impossible to set medical goals according to this parameter alone, but how criteria are set markedly affects the cost-effectiveness of treatment depending on the distribution of the HDL-C level and demographic composition of the target population; therefore, simulation involving these factors is one of the tasks that must be implemented to devise guidelines.

Fig. 1B shows the relationship between the LDL-C level and incidence (per 1,000 people) of myocardial infarction (lethal/non-lethal) in the JLIT, a cohort study that followed up a simvastatin-treated group for 5 years[8]. From this graph, the distribution of the HDL-C level in Japanese of corresponding ages (**Fig. 1A**)[9], and the population composition of the Japanese by age, the number of people needed to treat (NNT) and number of patients in whom the disease is prevented can be calculated when the control target is fulfilled 100% by reducing LDL-C (**Fig. 1C**). According to this calculation, the primary prevention efficacy, expressed as the inverse of NNT, is high at a target LDL-C level of 140 mg/dL but begins to fall rapidly as it is reduced to 120 mg/dL. Reflecting this, the incidence of myocardial infarction shows no further decrease when the target control level is set lower than 140 mg/dL. According to this analysis, roughly 140 mg/dL is considered to be medically and medicoeconomically appropriate as the target control level of LDL-C for primary prevention, at least on the basis of the results of the JLIT. In this case, the maximum preventive effect is 30-35% for myocardial infarction, which is in close agreement with the results of the MEGA study, the only large-scale interventional study of ischemic heart disease conducted in Japan using a statin[10].

Fig. 2B shows the decreases in the risk of ischemic heart disease associated with elevations of the HDL-C level in 3 epidemiological studies with prospective risk evaluation carried out in Japan including the JLIT[8, 11, 12]. While it is difficult to directly compare the incidences because the clinical definition of the endpoint varied among the studies, the peak decrease of the risk associated with increased HDL-C is less notable than that associated with the change of LDL-C in all studies. In other words, HDL-C-dependent decreases in the risk were observed even at HDL-C exceeding 60 mg/dL in all 3 studies. **Fig. 2C** shows the results of simulation similar to that of LDL-C performed using the results of the JLIT, which analyzed the therapeutic outcomes, on the basis of the HDL-C distribution curve in Japanese (**Fig. 2A**)[9] and the population composition. Since decreases in the risk associated with increases in HDL-C have not been directly demonstrated, the simulation was based on the hypothesis that increases in the risk associated with decreases in HDL can be reversed by increasing HDL-C. In contrast with the results concerning LDL, little decrease or peaking of the preventive efficacy associated with increased HDL-C was observed with an HDL-C level over 60 mg/dL. Reflecting this, the preventive effect against myocardial infarction could still be increased by raising the HDL-C level beyond 60 mg/dL. These results suggest that, under the hypothesis that the risk of myocardial infarction is reversibly reduced by elevating HDL-C, myocardial infarction can be prevented in 60-70% of the Japanese population at risk.

As far as these results are concerned, it can be concluded that the criterion of a "low HDL-C level" is unnecessary in guidelines for the control of HDL-C, and that the higher the HDL-C the better; however, according to the results in **Fig. 2A**, some studies have shown relatively large increases in the risk associated with decreases in HDL-C at about 50 mg/dL or below and, particularly, below 40 mg/dL; therefore, it may be reasonable to set a "caution level" around here. On the other hand, views on high HDL-C are divided. First, there is no epidemiological evidence indicating that higher HDL-C is better, even when it exceeds 60 mg/dL. This is probably because the population falling in this category is small (even though high HDL-C is relatively frequent in Japan) and cardiovascular incidence is low, making it difficult to obtain significant results.

In addition, the controversy is further complicated by the inclusion in this category of cases of homozygous CETP-deficient patients, in which elevations of HDL may not be considered to decrease the

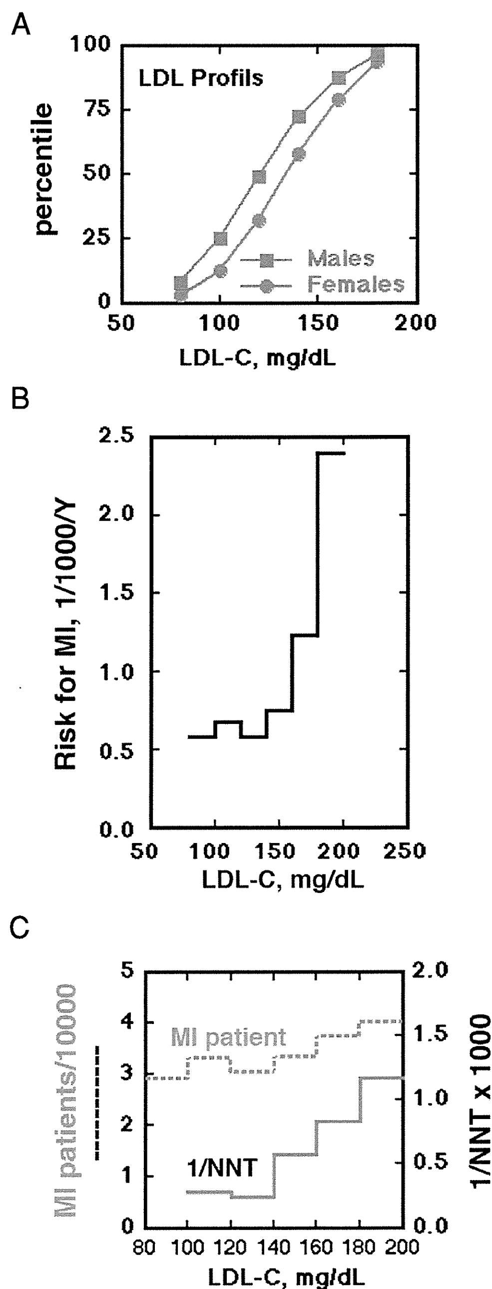

Fig. 1. Prevention of ischemic heart disease in Japanese by reducing LDL.

A: Distribution curve of the plasma LDL-C level in Japanese[9]. B: Relationship between the plasma LDL-C level and risk of "myocardial infarction" observed in the JLIT[8]. C: Simulation of the prevention of "myocardial infarction" based on Graphs A and B and demographic data for Japanese. Solid lines represent the inverse of NNT (·1,000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target LDL-C value at the left end of the segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when LDL is reduced to or lower than the level of the right end of the segment.

risk. The argument that increased HDL does not necessarily contribute to decreased risk is supported by the absence of a further decrease in the risk when the HDL-C increases above 70 mg/dL and the increased risk in patients with a homozygous CETP defect[13]; however, HDL-C is usually 80 mg/dL or higher and often reaches 100-200 mg/dL or even higher in patients with a homozygous CETP defect[13-16], and such high HDL-C should be considered separately from regular high HDL-C. Still, researchers are not in agreement concerning the increase in risk. In this sense, the differentiation of homozygous CETP deficiency is necessary in patients showing HDL-C exceeding 80 mg/dL, and there is no clinical or experimental evidence pointing to any conclusion about whether HDL-C should be maintained above this level. Nevertheless, the high prevalence of CETP deficiency among Japanese (1/20 for D442G and 1/100 for I14A) may have a limited but significant impact on the association between high HDL and atherogenesis in Japanese.

Proposal of Standards for Management of the HDL-C Level

On the basis of the above discussion, this article summarizes a proposal for the management of the HDL-C level as follows:

1) The evidence status is summarized as (1) A decrease in HDL-C is a strong risk factor for atherosclerotic disorders, particularly in Japan and, from the viewpoint of public health, it may be a more important risk factor than an increase in LDL-C; (2) While there are rational grounds for the argument that elevated HDL-C leads to a decreased risk, (3) there is as yet no direct evidence that elevating HDL-C is effective for the prevention and treatment of atherosclerotic disorders.

2) If elevations of HDL-C through interventional measures cause reversible decreases in the risk, this effect is expected, at least, up to 60 mg/dL or higher, and a simulation indicated that it eventually reduce the incidence of ischemic heart disease in Japan by 60-70%.

3) In risk management, high HDL-C is presently defined as 40 mg/dL or below. While there is no evidence that strongly urges a change in this definition, the results of epidemiological studies support "the higher the HDL-C level, the lower the risk," even in the "normal range" so that elevation of HDL-C may reduce the risk probably at least up to 70 mg/dL; however, there are no supportive data for this effect still being obtained over 80 mg/dL. Patients with a

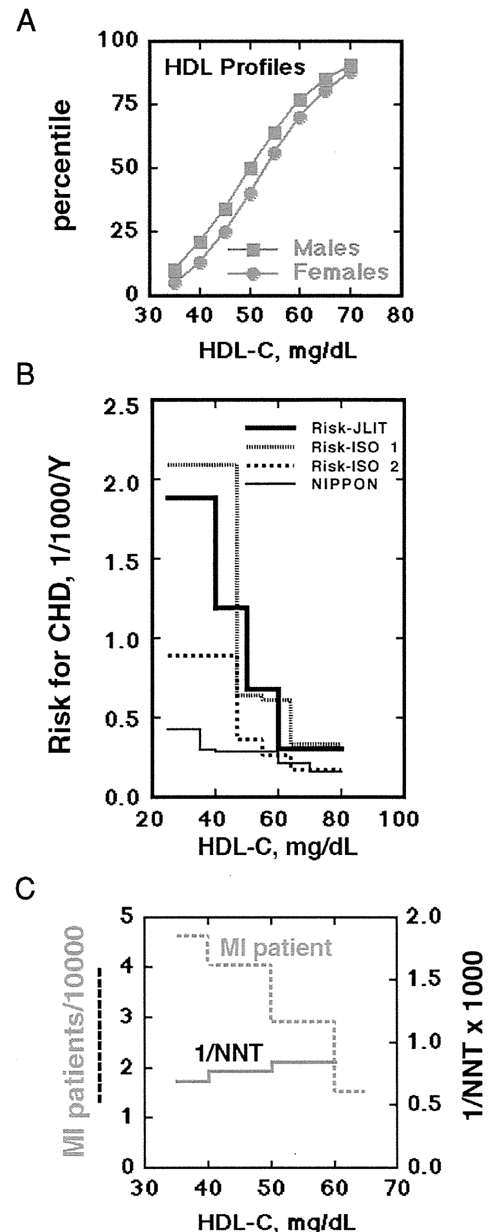

Fig. 2. Prevention of ischemic heart disease in Japanese by increasing HDL-C.

A: Distribution curve of the plasma HDL-C level in Japanese[9]. B: Relationship between the plasma HDL-CH level and risk of ischemic heart disease in Japanese. "Myocardial infarction" in the JLIT[8], "coronary artery disease" and "definitive diagnosis of myocardial infarction" by Kitamura, Iso, *et al.*[11], and "deaths due to cardiovascular diseases" according to NIPPON DATA[12]. C: Simulation for prevention of "myocardial infarction" based on Graphs A and B and demographic data of Japanese. Solid lines represent the inverse of NNT (x 1000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target HDL level at the right end of the horizontal segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when HDL is raised to the left end of the segment.

homozygous CETP deficiency should be followed-up while controlling other risk factors, not to dismiss the possibility of the risk increase with an extremely elevated HDL-C level. A gender-dependent strategy for HDL-C management should be discussed when further epidemiological and clinical evidence becomes available.

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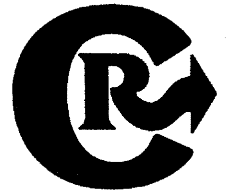

Combination of chondroitin sulfate and polyplex micelles from Poly(ethylene glycol)-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} block copolymer for prolonged in vivo gene transfection with reduced toxicity

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ABSTRACT

Nonviral polycation-based gene carriers (polyplexes) have attracted attention as safe and efficient gene delivery systems. Polyplex micelles comprised of poly(ethyleneglycol)-*block*-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-PAsp(DET)) and plasmid DNA (pDNA) have shown high transfection efficiency with low toxicity due to the pH-sensitive protonation behavior of PAsp(DET), which enhances endosomal escape, and their self-catalytic degradability under physiological conditions, which reduces cumulative toxicity during transfection. In this study, we improved the safety and transfection efficiency of this polyplex micelle system by adding an anionic polycarbohydrate, chondroitin sulfate (CS). A quantitative assay for cell membrane integrity using image analysis software showed that the addition of CS markedly reduced membrane damage caused by free polycations in the micelle solution. It also reduced tissue damage and subsequent inflammatory responses in the skeletal muscle and lungs of mice following *in vivo* gene delivery with the polyplex micelles. Subsequently, this led to prolonged transgene expression in the target organs. This combination of polyplex micelles and CS holds great promise for safe and efficient gene introduction in clinical settings.

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1. Introduction

Gene therapy has been explored for treating numerous diseases, including genetic disorders and cancers. Cationic polymers are often used for constructing nonviral gene carriers termed polyplexes, due to their advantages, such as large DNA loading capacity, ease of large-scale production, and reduced immunogenicity that has been an issue associated with viral vectors [1–3]. However, an inherent problem with using cationic polymers for gene transfection is their toxicity that causes tissue damage. The positively charged nature of polyplexes can induce nonspecific interactions with anionic biological molecules, such as blood cells, serum proteins, and extracellular matrices, which hinder the efficient delivery of genes, especially for *in vivo* applications.

A promising strategy to resolve these issues is to incorporate a hydrophilic protective layer of poly(ethylene glycol) (PEG) on the surfaces of polyplexes. A polyplex micelle system originally developed by our group is a good example of PEGylated polyplexes. In this system, PEG-polycation block copolymers are complexed with plasmid DNA (pDNA) to form a micellar structure that has a surface with hydrophilic and electrically neutral PEG and an inner core containing pDNA in a condensed state [4–8]. This structure increases the steric stability of the polyplexes under physiological conditions and exhibits less nonspecific interactions with biological components, such as serum proteins. In addition, a new polycation, poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), used as a core-forming segment of polyplex micelles, was developed to increase transfection efficiency [6].

Comprehensive analyses revealed that the high gene transfection capability of PAsp(DET) was mainly attributed to two distinct properties: (1) pH-responsive change in the degree of protonation, which enhanced endosomal escape [9] and (2) self-catalytic degradability under physiological conditions, which reduced the cumulative toxicity during transfection [10]. Using PEG-PAsp(DET) block copolymers, we achieved effective and sustained transgene expressions for *in vivo* local administration [11] and succeeded in bone

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regeneration by introducing osteogenic factor-expressing genes for bone defects in the mouse skull [8].

These PEG-PAsp(DET)/pDNA polyplex micelles (PMs) were applied to bone defect areas after their incorporation into a scaffold. Among several scaffolds tested, a commercial calcium phosphate paste (BIOPEX-R; Mitsubishi Pharma, Osaka, Japan) yielded the highest transgene expression. BIOPEX-R contains a considerable amount of chondroitin sulfate (CS), an anionic polycarbohydrate. This result led us to hypothesize that CS might play a critical role in gene introduction using PMs.

This study was designed to verify this hypothesis. We sought to identify the mechanisms involved with improved transfection after the addition of CS to PMs using physicochemical analyses, *in vitro* transfection, and *in vivo* local gene transfer.

2. Materials and methods

2.1. Materials

Plasmid DNA (pDNA) encoding luciferase (pGL4.13: Promega, Madison, WI) was amplified in competent DH5α *Escherichia coli* and purified using NucleoBond Xtra EF (Nippon Genetics, Tokyo, Japan). The pDNA concentration was determined from the absorbance at 260 nm. Dulbecco's modified Eagle's medium was from Sigma-Aldrich (St. Louis, MO, USA) and fetal bovine serum was from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Chondroitin sulfate A (CS) was from Sigma-Aldrich. Linear polyethyleneimine (LPEI) (Exgen 500; MW = 22 kDa) was from MBI Fermentas (Burlington, ON, Canada).

2.2. Animals

ICR mice (female, 7 weeks old) were purchased from Charles River Laboratories. All animal protocols were conducted with the approval of the Animal Care and Use Committee of the University of Tokyo.

2.3. Preparation of polyplex micelle solutions

PEG-PAsp(DET) block copolymer was synthesized as previously reported [6]. The PEG used had a molecular weight of 12,000 and the polymerization degree of the PAsp(DET) portion was determined to be 69 by ^{1}H-NMR. PEG-PAsp(DET) block copolymers and pDNA were separately dissolved in 10 mM Tris-HCl buffer (pH 7.4). PEG-PAsp (DET)/pDNA polyplex micelles (PMs) were obtained by simply mixing both solutions. After mixing for 10 min, PEG-PAsp(DET)/pDNA/CS polyplex micelles (CS-PMs) were prepared by adding CS at varying concentrations. In this study, the residual molar ratio of amino groups in PEG-PAsp(DET), phosphate groups in pDNA, and the total carboxyl and sulfate groups in CS was defined as N:P:CS. The final pDNA concentration was adjusted to 30 μg/ml for *in vitro* experiments and 133 μg/ml for *in vivo* experiments.

2.4. Luciferase expression and cell viability after transfection of PMs and CS-PMs

Cells were seeded at a density of 5000 cells/well in a 96-multiwell plate and cultured for 24 h. After the culture medium was replaced with fresh medium containing 10% fetal bovine serum, PMs or CS-PMs containing 0.18 μg pDNA was added to each well. Firefly luciferase expression was determined using a Luciferase assay system (Promega, Madison, WI, USA) and a GloMaxTM 96 microplate luminometer (Promega) following the manufacturer's protocol. Cell viability was determined using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's protocol.

2.5. Quantitative assay for the cellular uptake of pDNA

Cells were seeded at a density of 80,000 cells/well in a 6-multiwell plate and transfection was performed as in the previous section using 4 μg pDNA/well. Plasmid DNA was collected and purified from each well using a Wizard Genomic DNA purification kit (Promega). Purified DNA was then subjected to real time polymerase chain reaction (PCR) for the quantification of pDNA copies encoding Luc2 using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA) and the following primers: forward primer GGACTTGGACACCGG-TAAGA and reverse primer GTCGAAGATGTTGGGGTGTT. The copy number of β-actin was also determined by TaqMan Gene Expression Assays (Applied Biosystems) to normalize the cell number.

2.6. Evaluation of cell membrane integrity

Cells were seeded at a density of 10,000 cells/well in a 48-multiwell plate and cultured for 24 h. After the culture medium was replaced with PBS, PMs or CS-PMs containing 0.5 μg pDNA was added to each well. The cells were treated with 1 μM YO-PRO1 and 2.5 μg/ml Hoechst 33342 in phosphate buffered saline (PBS) 30 min later. Ten minutes later, the fluorescent intensity of each nucleus was quantitatively evaluated using a fluorescence microscope equipped with image-analysis software (IN Cell Analyzer 1000: GE Healthcare UK Ltd., Buckinghamshire, England).

2.7. Fluorescent labeling of PEG-PAsp(DET)

Alexa680-NHS (Invitrogen, Carlsbad, CA) was conjugated to the amino groups of PEG-PAsp(DET). PEG-PAsp(DET) was dissolved in 0.1 M $NaHCO_3$ at 4 °C, and an equimolar amount of Alexa680 in dimethylformamide (DMF) solution was added. After reacting for 3 h at 4 °C, the mixture was first dialyzed against an aqueous solution of 0.001 N HCl, and then against de-ionized water in dialysis tubing (MWCO: 12–14 kDa). The number of Alexa680 molecules conjugated to each PEG-PAsp(DET) molecule was determined to be 0.79 from the absorbance at 680 nm.

2.8. Fluorescence correlation spectroscopic (FCS) measurements of PEG-PAsp(DET)/CS mixtures

Alexa680-labeled PEG-PAsp(DET) solution at a concentration of 1 mg/ml and an equal volume of CS solution at varying concentrations was mixed and then diluted 30 times with PBS (pH 7.4) and MES buffer (pH 5.5) containing 20 mM MES and 150 mM NaCl, respectively. FCS measurements used a ConfoCor3 module (Carl Zeiss, Jena, Germany) equipped with a Zeiss C-Apochromat 40× water objective. A He–Ne laser (633 nm) was used for Alexa680-labeled pDNA excitation. For each sample, measurements were performed at room temperature with a sampling time of 20 s and repeated 10 times.

For the quantification of free PEG-PAsp(DET), a two-component model was applied to autocorrelation curves, where one component was free PEG-PAsp(DET) and the other was CS/PEG-PAsp(DET) complexes. When using this model, the diffusion times for free PEG-PAsp(DET) and CS/PEG-PAsp(DET) complexes were fixed. The diffusion time for CS/PEG-PAsp(DET) complexes was measured at CS:N = 1:1, because the diffusion time reached a plateau at a CS:N ratio of 0.5 at pH 7.4 and at a ratio of 1 at pH 5.5.

2.9. Förster resonance energy transfer (FRET) measurements

Double labeling of pDNA using fluorescein (ex/em = 492/518 nm) and Cy3 (550/570 nm) was done using Label IT Tracker Intracellular Nucleic Acid Localization Kits (Mirus) with a slightly modified protocol. The spectral properties of the pDNA were evaluated using a NanoDrop ND-3300 fluorospectrometer (NanoDrop Technologies,

Wilmington, DE) at an excitation of 470 nm with a blue LED. FRET efficiency was calculated from the relative emission ratio of Cy-3 (567 nm) to fluorescein (523 nm).

2.10. Size measurements of PMs by FCS

Plasmid DNA was labeled using Label IT Tracker Intracellular Nucleic Acid Localization Kits (Mirus, WI) following the manufacturer's protocol. The final DNA concentration was adjusted to 1.5 μg/ml. FCS measurements were performed as described previously. A He–Ne laser (633 nm) was used for Cy-5-labeled pDNA excitation.

2.11. Z-potential

Zeta potential was determined using a Zetasizer (Malvern Instruments, Worcestershire, U.K.) with a He–Ne Laser ($\lambda = 633$ nm) for the incident beam at a detection angle of 173° and at a temperature of 25 °C.

2.12. Intratracheal gene introduction into the lung

Mice were anesthetized by intraperitoneal administration of pentobarbital (60 mg/kg) (Kyoritsu Seiyaku, Tokyo, Japan). A micro-sprayer Model IA-1C-R (Penn Century, Philadelphia, PA) was placed into the trachea through the mouth, and then 50 μl of PM or CS-PM solution containing 6.7 μg pDNA was administered. Bronchoalveolar lavage (BAL) was performed with 500 μl PBS (instilled and recovered 4 times). LDH in BAL fluid was determined using a QuantiChrom Lactate Dehydrogenase Kit (BioAssay Systems, Hayward, CA) following the manufacturer's protocol. For mRNA measurements, lung tissue was extracted and total RNA was isolated using an RNeasy Mini Preparation Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Gene expression was analyzed by real-time quantitative PCR using TaqMan Gene Expression Assays and an ABI Prism 7500 Sequence Detector. An IVIS™ Imaging System (Xenogen, Alameda, CA) was used to evaluate luciferase expression in the lung after intravenous injection of D-luciferin following the manufacturer's protocol.

2.13. Hydrodynamic gene introduction into skeletal muscle

Hydrodynamic gene introduction into skeletal muscle was performed as previously reported [12]. Briefly, after anesthetizing mice with 3% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan), a tourniquet was placed on the proximal thigh to transiently restrict blood flow. Then, from a distal site of the great saphenous vein, naked pDNA, PM, or CS-PM solution (375 μl) containing 50 μg pDNA was injected in 5 s. At 5 min after injection, the tourniquet was released. Blood samples were collected from the *vena cava* and allowed to stand overnight at 4 °C, followed by centrifugation to obtain serum. Serum CPK was determined using an Enzychrom Creatine Kinase Assay kit (BioAssay Systems) following the manufacturer's protocol. An IVIS™ Imaging System was used to evaluate luciferase expression in muscle after intravenous injection of D-luciferin.

3. Results and discussion

3.1. In vitro transfection with chondroitin sulfate

Cultured cell lines were transfected by PEG-PAsp(DET)/pDNA polyplex micelles (PMs) in the presence of chondroitin sulfate (CS). As the optimized charge ratio of PEG-PAsp(DET): pDNA is N:P = 80:1 [6,8], PEG-PAsp(DET)/pDNA/CS polyplex micelle systems (CS-PMs) were constructed by adding CS to PM solutions at the charge ratios of PEG-PAsp(DET): pDNA: CS (N:P:CS) = 80:1:10 and 80:1:100, in which the CS charge was calculated as the total number of carboxylate and sulfate residues. As shown in Fig. 1A, CS-PMs induced higher transgene expression in NIH3T3 cells than PMs alone. Other cell lines also showed similar enhancements in transgene expressions by the addition of CS, although the optimal amount of CS differed depending on the cell line (Supplementary Fig. 1). Interestingly, although the final transgene expressions improved with CS, an evaluation of the time-dependent change revealed that CS addition induced a delay in the time to achieve maximal transgene expression, which was in good agreement with the delayed profile in cellular uptake of CS-PMs (Fig. 1B, C).

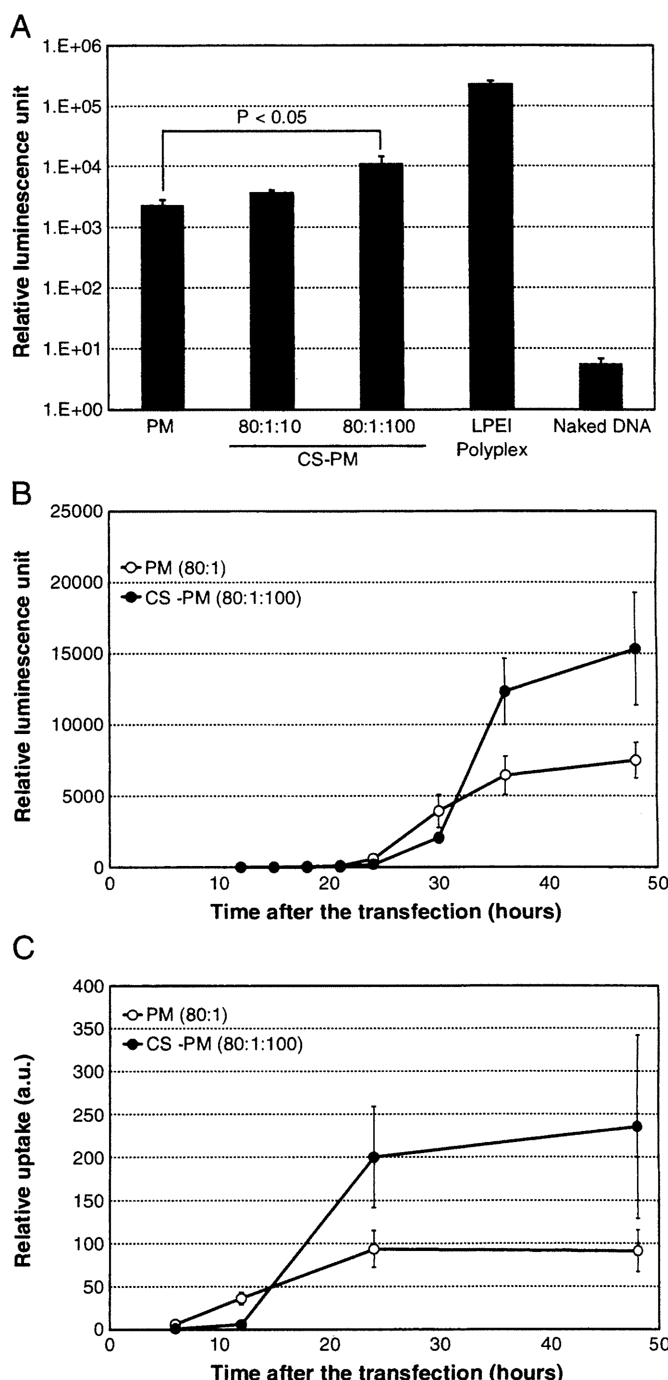

Fig. 1. Transfection efficiency in vitro. PM (N:P = 80:1), CS-PM (N:P:CS = 80:1:10 and 80:1:100), LPEI (N:P = 10:1) and naked DNA were transfected into NIH3T3 cells. A. Luciferase expressions after 48 h. B. Time-dependent profiles of luciferase expressions. C. Time-dependent profiles of pDNA uptake measured by quantitative PCR. Results are means ± SEMs (n = 5).

The PMs examined here, as well as most other polycation-based gene delivery systems, require an excess ratio of cationic polymers to pDNA for efficient transfection [6,8]. It is reasonable to assume that, at such a high N:P ratio, a considerable amount of PEG-PAsp(DET) exists in the free state without binding to PMs. Several reports have argued that free cationic polymers may be involved in endosomal escape and intracellular trafficking [13,14]. However, a critical issue is that these free polymers may induce toxic effects by interacting with cell membranes and other anionic biocomponents. It is possible that the anionic polycarbohydrate CS might abrogate such toxicity by associating with free polycations. Indeed, several recent studies that focused on the addition of polyanions, including CS, to polyplex systems showed enhanced transfection efficiency and reduced toxicity [15–20].

Accordingly, we analyzed the effect of CS on cell membrane integrity during *in vitro* transfection of PMs or CS-PMs. To assess cell membrane integrity, we used a DNA intercalator, YO-PRO1, which is impermeable to the normal cell membrane, but will permeate a membrane with perturbed integrity and emit a strong fluorescent signal due to DNA intercalation [21]. The fluorescent intensities of cultured cells due to permeated YO-PRO1 were quantitatively evaluated with a fluorescence microscope equipped with image-analysis software (IN Cell Analyzer 1000: GE Healthcare UK Ltd.).

At 30 min after transfection, the PMs formed at N:P = 80:1 induced an increase in fluorescent signals of YO-PRO1, suggesting enhanced cell membrane permeability (Fig. 2). In contrast, the PMs prepared at N:P = 3:1 showed almost no increase compared to the control. As reported previously, there was a substantial increase in the amount of free PEG-PAsp(DET) in the PM solution at an increased N:P [22]. Thus, it was reasonable to assume that the free PEG-PAsp(DET) in medium may have been involved in this significant increase in membrane permeability observed for the PM system at high N:P.

Indeed, addition of a corresponding amount of free PEG-PAsp (DET) into the medium induced as great an increase in fluorescence as the PMs prepared at N:P = 80:1, which was consistent with the previous assumption. Although polyplex from linear polyethyleneimine (LPEI) (N:P = 10), a commonly used transfectant, showed higher transfection efficiency than PMs or CS-PMs (Fig. 1A), the membrane damage and the cytotoxicity were notably observed for this system (Fig. 2 and Supplementary Fig. 2). In contrast, the membrane damage was substantially lowered in PEG-PAsp(DET) compared to PEI, indicating that the damage was sufficiently modest to have been effectively abrogated by the addition of CS. It should be noted that the enhanced cell membrane permeability was a transient, reversible phenomenon only at the initial phase of transfection, as it was not observed at 24 h after transfection (data not shown). Cell viabilities evaluated by an MTT assay at 48 h after transfection remained at nearly 100%, regardless of the presence or absence of CS (Supplementary Fig. 2).

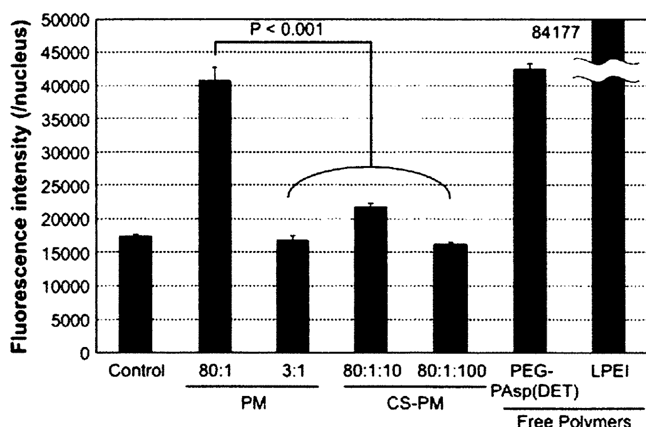

Fig. 2. Evaluation of membrane destabilization *in vitro*. PMs (N:P = 3:1 and 80:1), CS-PMs (N:P:CS = 80:1:10 and 80:1:100), and free PEG-PAsp(DET) and linear polyethyleneimine (LPEI)) with the same amine concentration as PMs (N:P = 80:1) were added to NIH3T3 cells, followed by treatment with YO-PRO1. The mean fluorescence intensity in cell nuclei was quantified for each well. About 3000 cells were analyzed in each well. Results are means of 3 wells ± SEMs.

3.2. Physicochemical characterization of CS-modulated polyplex systems

Next, we analyzed the effect of CS addition on the behaviors of free polycations as well as PMs. The reduced membrane damage after CS addition suggested that the amount of free polycations may have been substantially decreased in CS-PM solution due to the formation of polyion complexes between CS and excess PEG-PAsp(DET) in the medium.

Thus, to analyze the state of PEG-PAsp(DET) after mixing with varying ratios of CS, we measured the diffusion properties of Alexa680-labeled PEG-PAsp(DET) using FCS.

The diffusion time for PEG-PAsp(DET) reached a plateau at a CS:N ratio of 0.5:1, where the number of CS anionic charges was nearly equal to that of the PEG-PAsp(DET) cationic charges; approximately half of the amino groups in the 1,2-diaminoethane units in the side chain are protonated at pH 7.4 [9], suggesting the formation of a stoichiometric complex of CS and PEG-PAsp(DET) (Fig. 3). This result also suggested that there would be no free PEG-PAsp(DET) in the solution at CS:N ratios > 0.5.

The formation of a stoichiometric complex around CS:N = 0.5:1 was also confirmed by measuring the z-potential (Supplementary Fig. 3). While the complex prepared at CS:N = 0.5:1 had a single fraction at around a neutral charge, the complex at CS:N = 1:1 had another fraction around −30 mV corresponding to free CS. Further, DLS measurements revealed that the CS/PEG-PAsp(DET) complex that was formed had a size of 43 nm with a narrow distribution of polydispersity index (PDI) = 0.13, indicating the formation of polyion complex micelles with a CS/PAsp(DET) core surrounded by a PEG shell. Of note is that the CS-PM system, even without free polycations, yielded a higher transfection efficiency than PM alone, as shown in Fig. 1A. We will return to this matter in a subsequent section of this paper.

Next, we examined the physicochemical properties of PMs with or without CS. For this purpose, we used a charge ratio of N:P = 20:1, because too great an excess of cationic polymers may interfere with precise analyses of the PMs characteristics. Because CS has an anionic nature, there may be a possibility of loosening the condensed structure of PMs [23]. The condensed state of DNA was analyzed by FRET measurements using DNA labeled with a pair of donor–acceptor fluorescent dyes on a single DNA molecule [24,25]. Of interest is that even with the addition of CS, there was no significant change in the

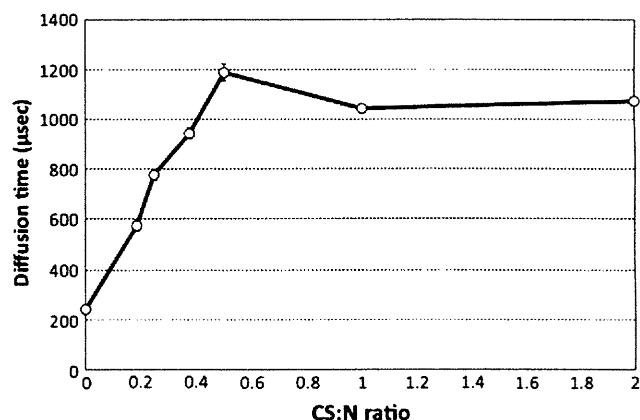

Fig. 3. Diffusion time measurements for Alexa680-labeled PEG-PAsp(DET) by FCS in the presence of varying amounts of CS in the solution. Results are means ± SDs.

condensation state of pDNA, suggesting that the disintegration of PM may not occur even in the presence of an appreciable amount of CS (Fig. 4). FCS using Cy5-labeled pDNA revealed that the diffusion time, which is proportional to particle diameter, showed a slight increase of approximately 1.3-fold after the addition of CS, suggesting some portion of CS may undergo the interaction with PMs (Fig. 5). The PMs z-potential became slightly negative after the addition of CS (+1.5 mV→ −5.5 mV). These results suggested that the interaction of CS caused slight changes in the diameter and z-potential of PMs, although the PM structure with condensed pDNA in the core remained stable.

3.3. In vivo gene delivery into lung and skeletal muscle

To confirm the effect of CS for *in vivo* conditions, we performed transfection into mouse lung via intratracheal administration. To evaluate tissue damage, we quantified LDH released into bronchoalveolar lavage fluid (BALF) at 30 min after the administration. As shown in Fig. 6, CS significantly reduced LDH release to a level comparable to that of untreated mice. We also assessed the consequent tissue damage by quantifying the mRNA levels of pro-inflammatory cytokines in lung tissue at 24 h after administration. Although PMs induced a low, but detectable upregulation of TNF-α and Cox-2, CS addition significantly reduced the production of these cytokines (Fig. 7). Interestingly, the luciferase expressions evaluated by an IVIS Imaging System were comparable for these two systems with or without CS (Fig. 8).

Another *in vivo* approach is by using a hydrodynamic injection that targets skeletal muscle [12]. To assess toxicity, serum creatine phosphokinase (CPK) was determined at 4 h after injection. Using CS-PMs, the CPK level was significantly reduced compared to PMs, suggesting that CS also reduced the tissue damage in muscles (Fig. 9). In addition, CS-PMs yielded a higher and more prolonged transgene expression compared to PMs and naked pDNA, although there was no statistically significant difference among these 3 groups (Fig. 10). These *in vivo* results indicated that CS exhibited the favorable effects of ameliorating tissue damage and reducing the inflammatory response at the injection site without affecting the transfection competency of PMs.

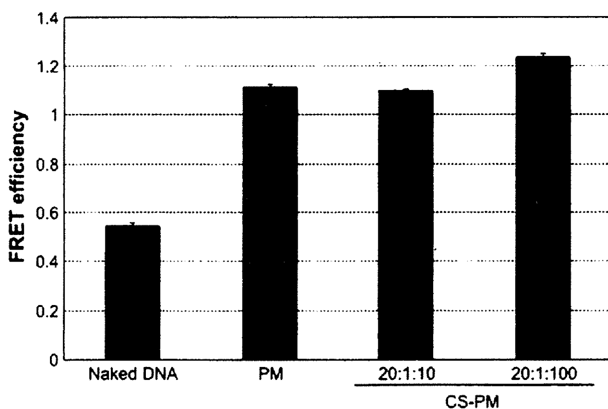

Fig. 4. Evaluation of pDNA condensation in the polyplexes by Förster resonance energy transfer (FRET). FRET efficiency between a pair of donor-acceptor fluorescent dyes (fluorescein and Cy3) tagged onto pDNA was determined for naked pDNA, PMs (N:P = 20:1), and CS-PMs (N:P:CS = 20:1:10 and 20:1:100). FRET efficiency is expressed as the emission intensity ratio of 567 nm (Cy3 emission) to 523 nm (fluorescein emission), where a higher value indicates a more condensed state of pDNA in the polyplexes. Results are means ± SDs (n = 3).

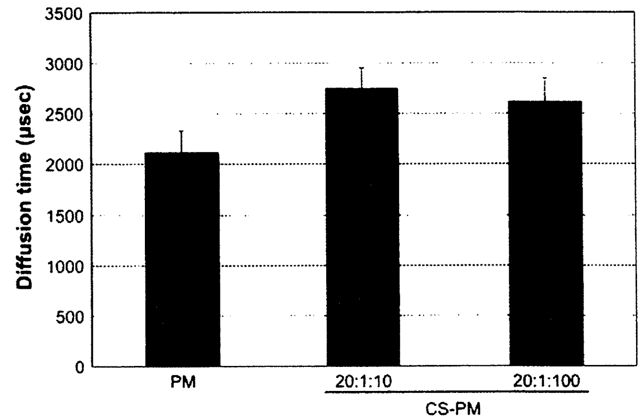

Fig. 5. Diffusion times for PMs (N:P = 20:1) and CS-PMs (N:P:CS = 20:1:10 and 20:1:100) measured by FCS. Results are means ± SDs.

3.4. Possible mechanisms for CS that improve the gene transfection efficacy of PM systems

The PM systems examined here consisted of a PEG-based block catiomer, PEG-PAsp(DET), which significantly reduced the cellular toxicity compared to polyplexes using non-PEGylated polycations [10,11]. Indeed, PM addition into the culture medium resulted in no decreases in cell viability as assessed by MTT assays (Supplementary Fig. 2). Nevertheless, a more sensitive assay to evaluate cell membrane integrity revealed that there was putative damage that increased membrane permeability, even though this was transient, and resulted in YO-PRO1 intercalated into chromosomal DNA to emit a fluorescent signal (Fig. 2). Worth noting is that this membrane damage was successfully abrogated by the addition of CS.

This was apparently due to neutralizing free polycations, the main component considered to cause membrane damage, in the medium via polyion complex formation. It is also worth noting that this propensity of CS to reduce membrane damage of the cultured cells caused by polycations is in line with the reduced adverse effects observed *in vivo*, including inflammatory cytokine production and tissue damage that induces LDH release. Thus, the use of CS in clinical settings is appealing for increasing the compliance of these PM systems. Although previous studies focused on the effects of anionic polycarbohydrates, such as hyaluronic acid (HA) and CS, on the behavior of cationic polylpexes [15–20], few of them addressed their roles in scavenging toxic free polycations.

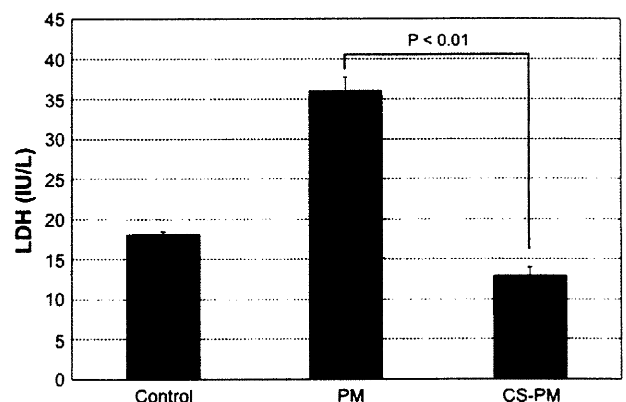

Fig. 6. LDH activity in bronchoalveolar lavage fluid (BALF). BALF was collected 30 min after the intratracheal administration of PMs (N:P = 20:1) and CS-PMs (N:P:CS = 20:1:100). Control indicates an untreated group. Results are means ± SEMs (n = 3).