women[3].

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

Aim

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

Methods

Subjects

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

Clinical Risk Factors

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

Analysis of Serum Lipids

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

Statistical Analysis

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

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

	Total subjects	CAD (+)	CAD (−)	*p value*
n	329	101	228	
Age (years)	43.8 ± 16.0	48.9 ± 10.2	41.6 ± 17.6	<0.001
Sex				
Men	139 (42.2%)	66 (65.3%)	73 (32.0%)	<0.001
BMI (kg/m²)	22.0±3.2	23.0 ± 2.7	22.6 ± 3.3	<0.001
Total cholesterol (mg/dL)	319 ± 70	333 ± 85	313 ± 61	0.039
Triglyceride (mg/dL)	(114) 80-176	(147) 96-193	(109) 76-162	0.263
HDL cholesterol (mg/dL)	50 ± 17	42 ± 14	54 ± 17	<0.001
LDL cholesterol (mg/dL)	241 ± 72	259 ± 84	232 ± 65	<0.001
Hypertension (*n*, %)	54 (16.4%)	33 (32.7%)	21 (9.2%)	<0.001
Diabetes Mellitus (*n*, %)	13 (4%)	8 (7.9%)	5 (2.2%)	0.014
Family history of CAD (*n*, %)	121 (36.8%)	46 (45.5%)	75 (32.9%)	0.028
Smoking habits (*n*, %)	127 (38.6%)	72 (71.3%)	55 (24.1%)	<0.001
Achilles tendon thickness (mm)	13.5 ± 5.4	16.2 ± 5.7	12.1 ± 4.6	<0.001
CAD present at first visit (*n*, %)	53 (16.1)	53 (52.5)	0 (0)	<0.001
Statin treatment at first clinic visit	39 (11.9)	18 (17.8)	21 (9.2)	0.541

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

Results

Patient Background

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

Onset of CAD

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

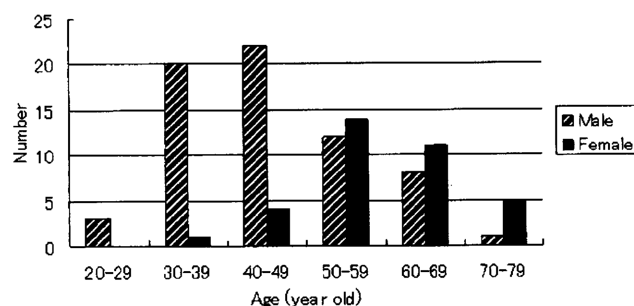

Fig. 1. Distribution of age when CAD was first identified in 101 men and women with heterozygous familial hypercholesterolemia (FH) and coronary artery disease (CAD), for the study period of 1969 to June 2007

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

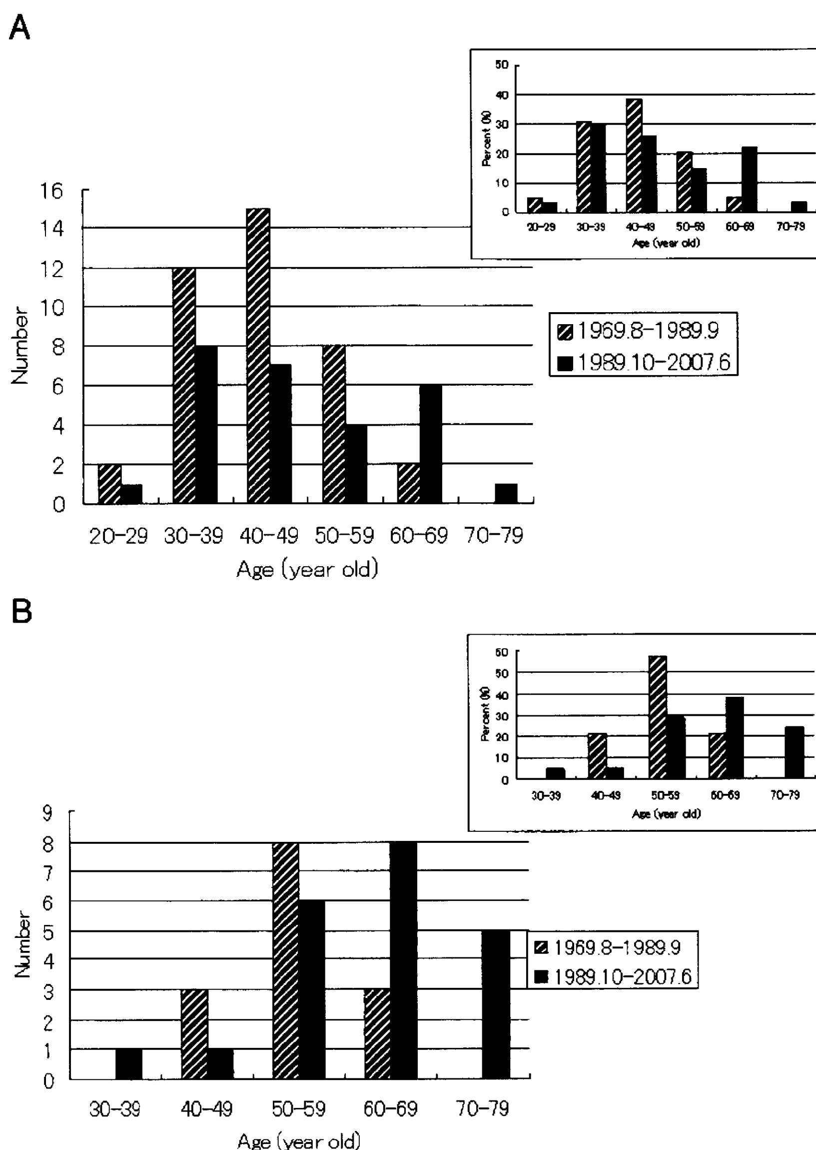

Fig. 3. Distribution of age at CAD onset in men (Panel A) and women (Panel B) who developed CAD before the end of September 1989 from October 1989

Each inset figure shows the percent of distribution, respectively.

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

Acknowledgments

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PCSK9：LDL受容体のあらたな制御機構と治療戦略

PCSK9：New LDL receptor regulator

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◎現在，脂質異常症の治療に対してはスタチン(HMG-CoA 還元酵素阻害剤)が第一選択薬として用いられ，優れた治療実績を残している．スタチンのおもな薬理学的機序はコレステロール合成系の遮断による細胞内コレステロールの枯渇であり，細胞はそれに対応すべく LDL 受容体の合成を促進し，血中よりコレステロールを回収・補充する．この結果として脂質異常が改善される．本機序におけるポイントは，“LDL 受容体の機能増強”であると考えることができ，脂質異常症に対する治療戦略として一般化できる．この観点からみればスタチンは間接的 LDL 受容体制御薬であるが，間接的であるがゆえに不要に多くの生体機能物質の生合成を阻害し，横紋筋融解症を代表とする重篤な副作用の発現を惹起している．LDL 受容体をより直接的に，かつ選択的に調節することが強力で副作用の少ない治療薬開発につながると考えられており，LDL 受容体を直接的に制御している因子の同定が進められている．

Key word：LDL受容体の制御，PCSK9，Idol

冠動脈疾患のリスクのなかでも LDL-コレステロール(LDL-C)値が重要であることは古くは Framingham Study において明らかになり，日本においても Nippon Data 80 などのコホート研究で明らかになっている．一方，スタチン(HMG-CoA 還元酵素阻害剤)の発見・開発に伴い，LDL-C 値の低下が可能になり，介入研究による LDL-C 低下療法の冠動脈疾患に対する一次・二次予防効果が示され，日本においても MEGA Study により一次予防効果が確認されている．冠動脈疾患の予防に対してスタチンのような“LDL 受容体制御薬”が非常に有効であることが示唆され，LDL 受容体制御機構の解明が進められている．

LDL 受容体の調節にかかわる因子として LDL 受容体のエンドサートーシスにかかわる“LDLRAP1/ARH”(LDLR adaptor protein 1)[1-3]や ADH(autosomal dominant hypercholesterolemia)の原因遺伝子としての“PCSK9”(Proprotein Convertase Subtilin/Kexin type 9)遺伝子[4-6]，また 2009 年には LDL 受容体のユビキチン化にかかわる“Idol”(inducible degrader of LDLRs)遺伝子[7]が発見され，あらたな LDL 受容体制御機構および創薬標的として注目を集めている．

そこで本稿では近年発見された新しい LDL 受容体制御分子，とくに“PCSK9”を中心に創薬のコンセプトや治療薬開発の現状について言及する．

細胞内コレステロールの調節

細胞内コレステロール濃度は多数の制御因子により巧みに調節されている．大別すれば 2 種の因子が中心的な役割を担っている．ひとつは細胞内のコレステロール合成および LDL 受容体の調節に転写レベルでかかわっている SREBP(sterol responsive element-binding protein)，もうひとつはコレステロールの異化，排出に関する LXR

(liver X receptor)である．

SREBP は小胞体膜に存在し，細胞内のコレステロールレベルのセンシングにかかわっているSCAP(SREBP cleavage activating protein)，S1P(Site-1 プロテアーゼ)，S2P(Site-2 プロテアーゼ)によるプロセシングを受け，膜から遊離し，核内へと移行する．ステロールの欠乏時にはこの活性化を受け，アイソフォームのひとつであるSREBP-2 はとくにコレステロール合成系のHMG-CoA 還元酵素や LDL 受容体遺伝子の転写を促進して細胞内コレステロール量は増加に傾く．一方，LXR は酸化ステロールをリガンドとしており，過剰のステロールに応答してコレステロールの異化，排出を促進して細胞内コレステロール量は減少に傾く．標的遺伝子としてコレステロール 7α 水酸化酵素，CETP(cholesterol ester transfer protein)，ABCA1, ABCG1 などが知られている．LXR は胆汁酸の合成を促進し，小腸のコレステロール吸収を阻害する．このように SREBP と LXR は相補的に細胞内コレステロールのホメオスタシスの維持にかかわっている．

スタチンは臨床でもっとも頻用されている脂質低下薬であり，コレステロール合成系の律速酵素である HMG-CoA 還元酵素を阻害することにより細胞内のコレステロールを枯渇させる．その結果，SREBP-2 が活性化を受け，合成系の酵素活性の上昇および LDL 受容体遺伝子の活性化により血中から細胞内へコレステロールを補充する．すなわち，スタチンの脂質低下効果には LDL 受容体遺伝子の活性化が鍵となっている．しかし，スタチンは LDL 受容体発現制御経路のかなり上流を阻害しており，多くの生体機能物質の生合成をも阻害してしまっているために横紋筋融解症を代表とする重篤な副作用の発現を回避できないともいわれている．そこで，より直接的に LDL 受容体を調節している因子に注目が集まっている．

PCSK9

PCSK9 におけるミスセンス変異は高 LDL-C 血症や低 LDL-C 血症に関連することが明らかとなっている．PCSK9 に“機能喪失型”変異を有する複合ヘテロ接合体患者においては血中 LDL-C 値が 14 mg/dl ときわめて低値を示し，PCSK9 が LDL-C 濃度の維持に重要な役割を果たしていることが明らかとなった[8]．PCSK9 はプロ蛋白質転換酵素ファミリーの 9 番目の因子であり，おもに肝，小腸，腎で発現している．PCSK9 は 3 種のドメイン(N 末端プロドメイン，触媒ドメイン，C 末端ドメイン)からなる約 74 kDa の可溶性酵素前駆体(proPCSK9)として合成され，細胞外へと放出される[9]．これら PCSK9 は Ca^{2+} 依存的に LRLR の細胞外 EGF-A ドメイン(epidermal growth factor-like repeat A domain)と相互作用し，エンドサイトーシスの形で細胞内へと取り込まれ LDL 受容体の分解を促進する[10]．エンドソーム内のような酸性条件下では PCSK9 が LDL 受容体の構造変化を妨げ，LDL 受容体を分解へと誘導するものと考えられている．

PCSK9 ノックアウトマウスはワイルドタイプのマウスに比較して血中 LDL-C 値が約半分まで低下しており，PCSK9 阻害薬の有効性が示唆されている．さらに，このノックアウトマウスではスタチンに対する感受性が大きく亢進しており，PCSK9 阻害剤とスタチンの併用療法の可能性についても言及されている[11]．PCSK9 阻害薬の開発は大きく分けて 4 つのアプローチで押し進められている．①小分子を用いた手法，②抗体医薬を用いた手法，③アンチセンス核酸を用いた手法，④siRNA を用いた手法である．以下，各アプローチについて概説する．

① 小分子

Berberine は漢方生薬に含まれる有効成分として有名であり，多くの疾患に対する適応がある．脂質代謝に関しては LDL 受容体 mRNA の安定化および PCSK9 mRNA の転写抑制効果が報告されており，脂質異常症患者 32 人に 3 カ月間 Berberine を経口投与した場合，LDL-C 値が約 25%低下することが見出されている[12,13]．

小分子化合物を用いるアプローチは Berberine 以外にはほとんど報告されていない．小分子化合物はシーズの探索が一般的に困難であり，分子設計，スクリーニング用の化合物ライブラリや適切な薬効評価系などを必要とする．とくに PCSK9 と LDL 受容体はたがいに接するように相互作用

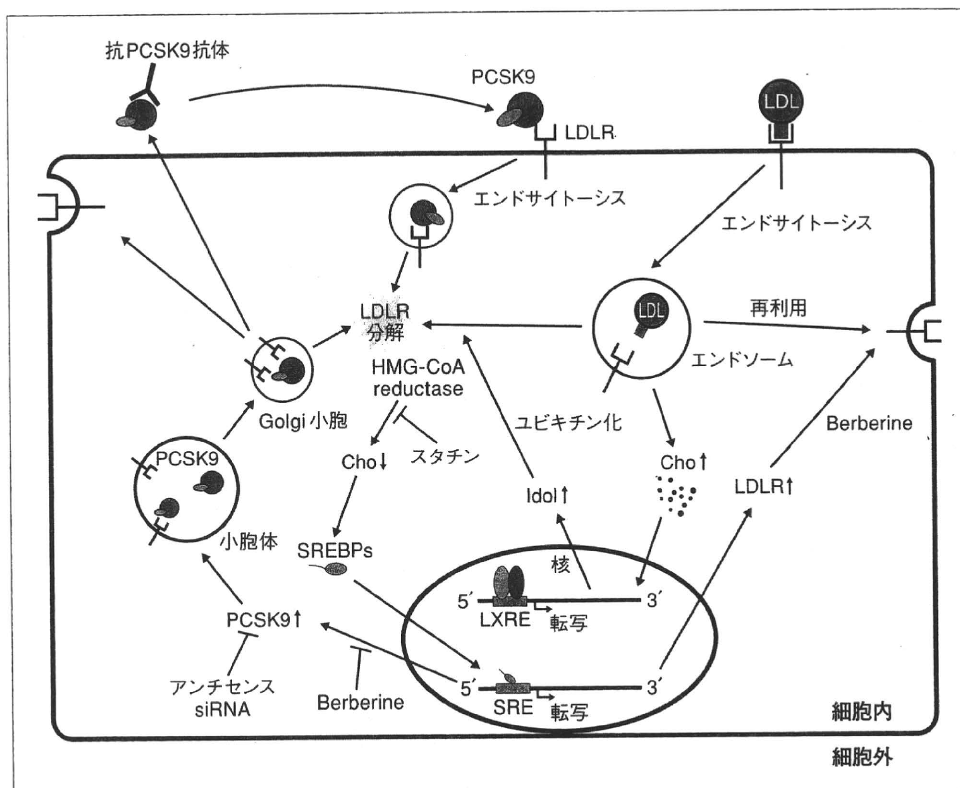

図 1　LDL受容体の制御機構

LDL 受容体はコレステロール合成系の制御にかかわる SREBPs および LXR により制御を受けている．PCSK9 および Idol はこれらの転写因子の制御下で直接的に LDL 受容体を分解する．
LDLR：LDL 受容体．

しているため，合理的な小分子の設計が困難だといわれている．

② 抗体医薬

抗 PCSK9 抗体については Amgen 社の Chan らにより開発が進められている[14]．彼らが開発した mAb1 は PCSK9 と相互作用することにより LDL 受容体の EGF-A ドメイン/PCSK9 の複合体形成を立体的に妨害する．サルに投与した場合には単回投与で最大 LDL-C 値が 80%低下し，作用は 10 日間持続したと報告している．

③④ 核酸医薬

ISIS pharmaceutics 社の Graham らは体内での分解に耐えるように化学修飾された DNA 鎖を用い，マウスの PCSK9 mRNA を効果的に抑制することに成功している．彼らは高脂肪食負荷マウスに対してアンチセンス核酸を投与し，肝の PCSK9 mRNA を 92%，TC 値を 53%，LDL-C 値を 38%低下させた[15]．同社が開発中の PCSK9 を標的としたアンチセンス "BMS-PCSK9RX" に関しては，現在，前臨床試験の段階にある．そのほか，Santaris Pharma 社でも同様に，LNA とよばれる標的 mRNA の転写を高効率に抑制する人工 DNA を用いて PCSK9 に対するアンチセンス医薬の開発に取り組んでいる．さらに，Alnylam Pharmaceutics 社の Frank-Kamenetsky らは，siRNA を脂質担体に吸着させ，その複合体をサルの静脈より投与し，LDL-C 値の良好な低下を確認している[16]．

このように多くの製薬会社や研究機関が PCSK9 阻害剤の開発に着手しており，おおむね良好な結果を得ている．現在のところ先述した理由から小分子の新規開発は難しいと考えられており，抗体医薬および核酸医薬を用いたアプローチが注目を集めている．

おわりに

PCSK9 阻害薬のコンセプトから阻害薬開発の現状について概説した(図 1). PCSK9 は SREBP-2 により制御されており，LDL 受容体と同期して発現することにより LDL の取込みを制限している．このため PCSK9 は，血中 LDL-C 濃度の維持に大きな寄与があり，スタチンとの併用療法だけでなく，単剤での有効性も期待できる．

LXR に関しては，アゴニストは ABCA1 や ABCG1 などを活性化し，コレステロールの異化や排出を促進する一方で，SREBP-1c を誘導し，高トリグリセリド血症を惹起することが知られている．このため，LXR に対する薬剤には長所と短所が両立しており，臨床応用には副作用に留意すると同時に，より選択的なアゴニストの開発が必要となっている．他方で LXR が LDL 受容体の発現にかかわっていることが確認されている．LXR は，Idol とよばれる E3-ubiquitin ligase を発現上昇させ，LDL 受容体をユビキチン化することにより LDL 受容体を蛋白レベルで調節している．Idol を標的とした薬剤は LDL-C 値を低下させる効果が期待できるが，その薬効の選択性については今後検討の余地がある．また，PCSK9 と同様に，LDL 受容体を増加させるスタチンと Idol 阻害剤の併用により相乗的な脂質低下効果が期待できる．

このように LDL 受容体の発現調節にかかわる因子の発見が，より副作用の少ない脂質異常症改善薬の開発につながるものと期待されている．

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ARHとPCSK9

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ARHとPCSK9

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はじめに●

家族性高コレステロール血症(FH)は，高LDLコレステロール血症，皮膚および腱黄色腫，若年性動脈硬化症による冠動脈疾患を特徴とする遺伝病である．Goldstein，Brownらにより，LDL受容体遺伝子が原因遺伝子として同定された．近年，高LDLコレステロール血症や黄色腫など，FHと同様の病態を示すがLDL受容体に異常がない家系の解析から，LDL受容体のアダプター蛋白であるlow density lipoprotein receptor adaptor protein 1(LDLRAP1)やセリンプロテアーゼであるproprotein convertase subtilsin/kexin-type 9(PCSK9)が同定され，LDL代謝経路にかかわり，血中LDLコレステロール値の制御にかかわることがわかってきた．本項では，これらの遺伝子の機能とLDLコレステロール値制御機構について概説する．

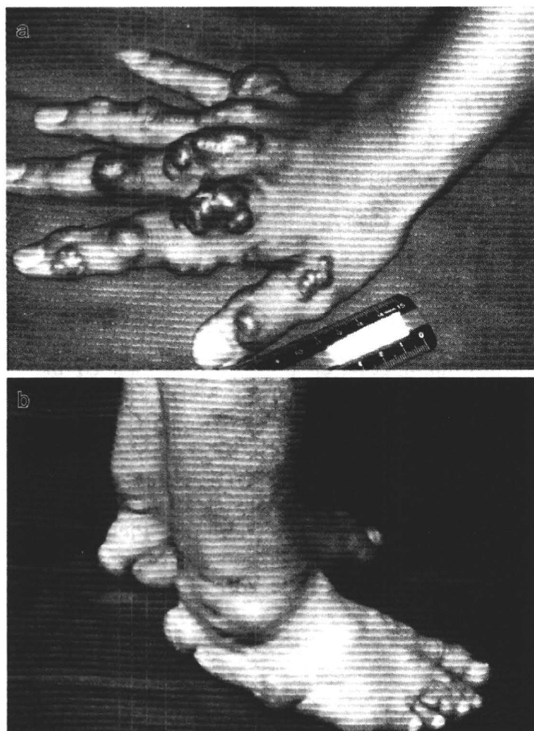

図1　ARH患者の巨大な皮膚黄色腫の写真
a　手背の皮膚および腱黄色腫
b　アキレス腱，足指腱の肥厚
（文献4)より引用）

ARH●

1. ARHの発見

われわれは血清TC値が500～600 mg/d*l*，多発性皮膚黄色腫，アキレス腱肥厚，若年性動脈硬化症を伴い，臨床的にはFHホモ接合体であると考えられたが，皮膚線維芽細胞のLDL受容体活性に異常を認めなかった姉弟例を報告した[1]．2001年，Garciaらは6家系からポジショナルクローニングによりLDL受容体のアダプター蛋白であるLDLRAP1遺伝子を同定し，常染色体劣性遺伝性高コレステロール血症autosomal recessive hypercholesterolemia(ARH)と名づけられた[2]．

2. ARHの特徴

ARHは高LDLコレステロール血症，黄色腫，若年性冠動脈疾患を示すなど，臨床徴候がLDL受容体遺伝子異常により引き起こされる家族性高コレステロール血症(FH)ホモ接合体に非常に似ているにもかかわらず，脂質降下療法の反応がやや良いことが知られている．エゼチミブが有効であるとの報告もある．黄色腫は，FHに比べて巨大である例が多い(**図1**)．

3. ARH遺伝子改変マウスによる検討

ARH KOマウスにおいて高LDLコレステロール血症を認め，LDL代謝回転の遅延を認めること，肝臓でのLDLの取り込みが遅延することからも，ARHがLDL受容体の取り込み機能に必須であることが裏づけられた[3]．

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●常染色体劣性遺伝性高コレステロール血症(ARH)は，LDL 受容体のアダプター蛋白(LDLRAP1)をコードする遺伝子の変異によるものである．
●セリンプロテアーゼである PCSK9 は，LDL 受容体の分解にかかわっており，高コレステロール血症治療のターゲット分子として注目されている．

PCSK9 ●

1. PCSK9 について

1999 年，Verret らは LDL 受容体，apoB ともに異常のない常染色体優性遺伝形式をとる高脂血症の 13 家計を報告し，それを FH3 と呼んだ．2003 年，Abifadel らによりポジショナルクローニングが行われ，PCSK9 遺伝子が同定された[4]．

2. PCSK9 の機能

PCSK9 は proteinase K ファミリーの一つであり，神経細胞のアポトーシスを制御する NARC-1 (neural apoptosis-regulated convertase 1) をコードするとされていた．PCSK9 は LDL 受容体の分解にかかわる分泌蛋白であり，その機能上昇変異により肝臓における LDL 受容体の蛋白量が減少し，高 LDL コレステロール血症をきたすこと，さらに機能低下変異により，低 LDL コレステロール血症をきたすことが知られている．

3. PCSK9 遺伝子変異による ADH の臨床像

PCSK9 は LDL 受容体の分解にかかわり，その発現増加により肝臓における LDL 受容体の蛋白量が減少し，高 LDL コレステロール血症をきたす．D374Y 変異を有するイギリス人の 4 家系の報告では[5]，FH ヘテロ接合体よりも若年齢で高コレステロール血症，スタチン治療への抵抗性を示していた．一方，S127R，F216L 変異を持つフランス人の報告ではコレステロール値はさほど高値を示さない．

わが国においては，LDL 受容体および apoB 遺伝子変異を伴わない臨床的 FH 患者における PCSK9 遺伝子変異の検討が金沢で行われているが，低頻度であると報告されている．

4. PCSK9 を標的とした高コレステロール血症治療薬

PCSK9 が機能低下変異により低コレステロール血症を示すことから，次世代の高コレステロール血症治療薬のターゲットとしてアンチセンスや siRNA などの核酸医薬の開発がなされており，臨床試験が進行中である．

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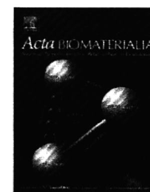

Stable modification of poly(lactic acid) surface with neurite outgrowth-promoting peptides via hydrophobic collagen-like sequence

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A B S T R A C T

Surface modification of poly(DL-lactic acid) (PLA) scaffolds has been performed using a biofunctional small peptide composed of collagen-like repetitive sequence and laminin-derived sequence (AG73-G_3-$(PPG)_5$) via hydrophobic interaction. The results of surface analysis suggest that AG73-G_3-$(PPG)_5$ can be stably adsorbed onto PLA films via hydrophobic interaction at the $(PPG)_5$ region, and form an extracellular matrix-like layer composed of both structural and biosignalling sequences. In addition, neurite outgrowth of PC12 cells was observed on the AG73-G_3-$(PPG)_5$-adsorbed PLA film. These results indicate that AG73-G_3-$(PPG)_5$ very effectively enhances neurite outgrowth activity on PLA films. The hydrophobic adsorption of collagen-like peptide bound to biosignalling molecules may be widely applied as a surface modifier of PLA films for tissue engineering.

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1. Introduction

Tissue engineering has been proposed as an approach to replace damaged, injured or missing tissue with biologically compatible substrate combining cells or biosignalling molecules and scaffolds [1,2]. Scaffolds assume the role of a temporary extracellular matrix (ECM) where biodegradability and biocompatibility are essential for tissue regeneration. Furthermore, biodegradable scaffolds should be designed not to obstruct tissue regeneration via cell-induced natural healing. The cellular responses to the scaffold surfaces determine whether tissue regeneration will be promoted or obstructed. Therefore, it is very important to control the biological property of the scaffold surfaces [3].

Poly(lactic acid) (PLA) is widely used for biodegradable scaffolds as it possesses a number of suitable characteristics for this role. PLA can be hydrolytically degraded into lactic acid; this degradation requires only water, and the final product can immediately be metabolized in vivo [4]. Moreover, PLA material exhibits excellent shaping and molding properties because of its mechanical versatility. However, insufficient interaction between PLA materials and cells leading to in vivo foreign-body reactions is a major problem because the required biological activities are not inherent in PLA. PLA lacks functional groups and so cannot be easily modified with bioactive molecules. Therefore, many investigators have attempted to impart functional groups to PLA in order to enhance its biological activity by using copolymerization or chemical grafting with other polymers [5], plasma treatment [6], chemical modification [7] and physical adsorption. In previous studies, we reported on the preparation of poly(lactic-co-malic acid)-conjugated Arg-Gly-Asp (RGD) tripeptide [8] and gelatin-immobilized PLA scaffold [9] in order to improve the cell attachment. However, because these techniques are prone to adverse chemical reactions, it is necessary to develop techniques that are simpler and offer better biocompatibility. Physical adsorption, which is driven by electrostatic, hydrophobic and specific interactions, has been noted as a simpler surface modification technique of PLA scaffolds [10–12].

In the current work, neurite outgrowth-promoting peptides, consisting of laminin-derived sequence and collagen-like sequence, were designed as surface modifiers of PLA films via hydrophobic adsorption. PLA is preferred as a base material for a nerve regeneration conduit because of its excellent shaping and molding properties [13]. However, PLA does not inherently cater to any nerve regeneration activity. If biologically modified PLA-based artificial nerve can promote nerve regeneration, it might be possible to avoid donor site defects in autologous nerve transplantation. It was reported that laminin-derived sequence AG73 supports neurite outgrowth [14], and therefore was selected as a nerve-regenerating peptide.

On the other hand, it is well known that collagen is a predominant component of ECM [15,16]. The major part of collagen

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consists of Xaa-Yaa-Gly repetitive sequences, where Xaa and Yaa positions are often occupied by Pro and 4(R)-hydroxyproline (Hyp), respectively, and forms the hydrophobic polyproline-II (PP-II) structure [17–20]. The collagen triple-helix structure is composed of three PP-II chains, and collagen-like peptides (CLPs) such as $(Pro-Pro-Gly)_n$ are also able to form a triple-helix structure [21–23]; therefore, CLP is expected to be adsorbed by PLA films via hydrophobic interaction. Animal-derived collagen has also been intensively investigated as a conduit for nerve regeneration because of its high bioactivity [24]. However, animal-derived collagens possess high antigenicity in vivo because of the unnecessary biosignal sequences and enzymatically digested fragments [25]. CLP, which is the repetitive sequence at a structural region of collagen without any enzyme-digestible sequence, is anticipated to be of low immunogenicity.

Here, we are reporting on a neurite outgrowth-promoting peptide composed of AG73 and CLP ($AG73-G_3-(PPG)_5$) as a surface modifier of PLA films for tissue engineering. Conformation of $AG73-G_3-(PPG)_5$ was studied by circular dichroism (CD) spectroscopy. The surface characteristics of $AG73-G_3-(PPG)_5$-adsorbed PLA film were investigated by water contact angle measurement and X-ray photoelectron spectroscopy (XPS). PC12 cells were primed with nerve growth factor (NGF) and cultured on the $AG73-G_3-(PPG)_5$-adsorbed PLA films, and the neurite outgrowth activity was then quantified.

2. Materials and methods

2.1. Materials

$(PPG)_{10}$, AG73 (RKRLQVQLSIRT) and $AG73-G_3-(PPG)_5$ were commercially synthesized by SCRUM, Inc. (Tokyo, Japan). PLA (Mw 130,000) was obtained from Mitsui Chemicals, Inc. (Tokyo, Japan). Progesterone, sodium selenite (N_2SeO_3) and transferrin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). NGF and horse serum (HS) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Insulin, advanced DMEM/F12 and penicillin–streptomycin were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from MP Biomedicals, Inc. (Solon, OH, USA).

2.2. Methods

2.2.1. Circular dichroism

CD spectra were measured by a J-720 spectropolarimeter (Jasco Co., Tokyo, Japan) with a standard analysis program. The temperature was controlled using a recirculating waterbath and spectra was recorded with a 0.1 cm path length cell, using a scanning speed $10\ nm\ min^{-1}$, with a 1.0 nm spectral bandwidth, over the wavelength range from 190 to 250 nm. Peptides were dissolved with water at 0.25 mM. Data are represented in molar elipticities ($[\theta]\ deg\ cm^2\ dmol^{-1}$).

2.2.2. Peptide adsorption on PLA films

PLA films (diameter $\varphi = 6.0$ mm; $t = 0.5$ mm) were prepared with a hot shrinking machine at 180 °C and sterilized by UV irradiation. Three peptides, $(PPG)_{10}$, AG73, and $AG73-G_3-(PPG)_5$, were dissolved in sterilized water at 10 µM, and then 1 ml of each peptide solution was poured onto a PLA film in a 24-well cell culture plate. Peptide solutions were dried for 24 h. In order to get rid of any excessively adsorbed peptide, the PLA films were washed with 1 ml of sterilized H_2O or 1.0 M NaCl aqueous solution twice for 30 min, and then the films were washed with 1 ml of sterilized H_2O again and dried in vacuo.

2.2.3. Water contact angle

The contact angle with distilled water was measured by using a contact-angle meter (CA-X; Kyowa Interface Science Co., Ltd., Saitama, Japan). Images of the water spreading on the sample were recorded by a camera and then analyzed. Three samples were measured for each group.

2.2.4. X-ray photoelectron spectroscopy

The surface composition of peptide-adsorbed PLA films was determined using an ESCA-3400 (Shimadzu Co., Kyoto, Japan). The X-ray source was a monochromatic Mg K_α X-ray from a rotating anode. Survey scans were measured from 0 to 1200 eV. Peak positions and areas were analyzed and ratios for C1s, N1s and O1s were calculated by using software provided by the manufacturer.

2.3. Cell culture

Rat adrenal pheochromocytoma PC12 cells (RIKEN BioResource Center, Ibaraki, Japan) were maintained in DMEM supplemented with $100\ U\ ml^{-1}$ penicillin, $100\ \mu g\ ml^{-1}$ streptomycin, 10% FBS and 7.5% HS. PC12 cells were cultured in poly-D-Lys coated cell-culture dishes (BD, NJ, USA) and maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air.

2.4. Neurite outgrowth assay

The neurite outgrowth assay was performed by using PC12 cells as the model of neural stem cells [26]. PC12 cells were primed with $100\ ng\ ml^{-1}$ NGF for 24 h on polystyrene cell-culture dishes. The cells were then collected by agitation and placed in the culture medium for 30 min at 37 °C in an atmosphere of 5% CO_2 and 95% air. The cells were washed and resuspended with advanced DMEM/F12 containing $5\ \mu g\ ml^{-1}$ insulin, $100\ ng\ ml^{-1}$ NGF, 20 nM progesterone, 30 nM Na_2SeO_3 and $100\ mg\ ml^{-1}$ transferrin. The cells were then seeded on peptide-adsorbed PLA films at a seeding density of 2.0×10^4 cells $film^{-1}$ in 24-well cell culture plates, and incubated at 37 °C for 24 h. PC12 cells on peptide-adsorbed PLA films were fixed with 10% formalin and stained by 4% crystal violet/methanol solution, and then the number of PC cells with or without neurites was determined in order to evaluate the neurite outgrowth activity as described elsewhere [27,28]. The lengths of neurites were measured using software (Image J; National Institute of Mental Health, MD, USA) [29]. Cells with neurites longer than 50 µm and those with neurites shorter than 50 µm were counted separately.

3. Results and discussion

3.1. Secondary structure of peptides

The CD spectra of $(PPG)_{10}$, AG73 and $AG73-G_3-(PPG)_5$ are shown in Fig. 1. The CD spectrum of $(PPG)_{10}$ in water at 37 °C exhibited a strong negative band at 209 nm and a positive band at 229 nm, which are known as typical patterns of collagen triple-helix and PP-II structure [20,30]. Although the CD spectrum of PP-II is similar to that of collagen triple-helix, the transition temperature of $(PPG)_{10}$ is reported to be about 28 °C in water [31]. Therefore, this CD spectrum indicates that $(PPG)_{10}$ forms the PP-II structure. The CD spectrum of AG73 showed a strong negative band at 199 nm, assigned as a random-coil structure. In the case of $AG73-G_3-(PPG)_5$, the CD spectrum indicated an intermediate pattern between $(PPG)_{10}$ and AG73. This means that a negative band was blue-shifted and a positive band at 229 nm was decreased in comparison with $(PPG)_{10}$. In addition, all CD spectra have an isosbestic

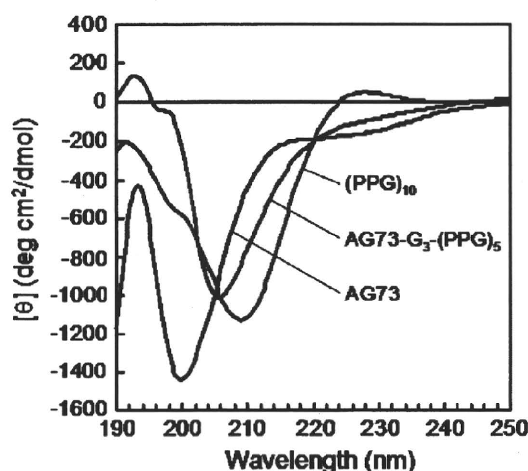

Fig. 1. Circular dichroism spectra of $(PPG)_{10}$, AG73 and AG73-G_3-$(PPG)_5$ in water.

point at 220 nm. The results suggest that the CLP region of AG73-G_3-$(PPG)_5$ also forms the PP-II structure. It is well known that the PP-II structure is the predominant secondary structure in collagen triple-helix. In addition, the PP-II structure of $(PPG)_n$ repetitive sequence exposes hydrophobic five-membered rings of Pro residues. Therefore, it is inferred that $(PPG)_n$ is adsorbed onto PLA films via hydrophobic interaction.

3.2. Surface characterization of peptide-adsorbed PLA films

To compare the hydrophilicity of the peptide-adsorbed PLA film surfaces, the water contact angle was measured (Table 1). The water contact angle of non-adsorbed PLA films was 79.1 ± 1.2°, which indicates a hydrophobic surface. After adsorption of $(PPG)_{10}$, AG73 and AG73-G_3-$(PPG)_5$, the water contact angle changed to 70.1 ± 3.9°, 31.2 ± 2.1° and 55.6 ± 1.9°, respectively. The water contact angle drastically decreased with AG73 and AG73-G_3-$(PPG)_5$ adsorption, but with $(PPG)_{10}$ adsorption the decrease was considerably less. It seems that the surface wettability of peptide-adsorbed PLA films corresponded to the hydrophilicity of the peptides, i.e. $(PPG)_{10}$ is more hydrophobic than AG73 and AG73-G_3-$(PPG)_5$. Safinia et al. reported that the PLA film surface shows a negative ζ-potential at physiological pH [32] because of a carboxyl group at the terminal of the PLA molecule. Therefore, the results suggest that these peptides are adsorbed onto PLA film via hydrophobic or electrostatic interactions.

The XPS N1s spectrum is shown in Fig. 2. The XPS spectra of $(PPG)_{10}$-, AG73- and AG73-G_3-$(PPG)_5$-adsorbed PLA films exhibited a N1s peak corresponding to amino acids. In order to compare the adsorbed peptide ratio on PLA films, the elemental ratios are summarized in Table 2. N1s was not detected clearly because PLA itself does not contain nitrogen. The N1s/C1s ratios were 0.07, 0.09 and 0.18, and the N1s/O1s ratios were 0.03, 0.04 and 0.09. The N1s/C1s and N1s/O1s ratios were increased with peptide adsorption; in particular, the highest values were shown for AG73-G_3-$(PPG)_5$. The AG73-G_3-$(PPG)_5$ might be adsorbed at high density via hydrophobic interaction between $(PPG)_5$ region and PLA. The reason for the decrease in water contact angle of AG73-G_3-$(PPG)_5$-adsorbed PLA film (Table 1) is that the hydrophilic AG73 region in AG73-G_3-$(PPG)_5$ was partially exposed to solution. Ji et al. also indicated that the poly(ethylene oxide–propylene oxide–ethylene oxide) amphiphilic triblock copolymer bearing RGD tripeptides adsorbed onto PLA films through hydrophobic interaction, and its hydrophilic regions were exposed to solution phase [11]. As a result, the AG73-G_3-$(PPG)_5$ adsorbs via hydrophobic interaction and constructs an ECM-like layer on the PLA film surface.

Table 1
Water contact angle of peptide-adsorbed PLA films.

	CA (°)
Non-adsorbed	79.1 ± 1.2
$(PPG)_{10}$	70.1 ± 3.9
AG73	31.2 ± 2.1
AG73-G_3-$(PPG)_5$	55.6 ± 1.9

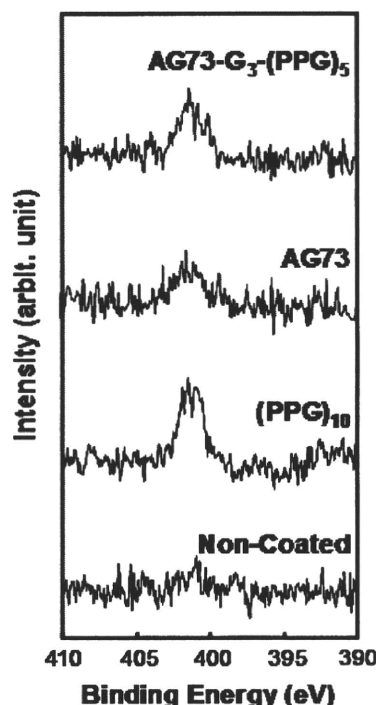

Fig. 2. XPS spectra of the N1s region of peptide-adsorbed PLA films.

Table 2
Elemental ratio of nitrogen to carbon (N1s/C1s) or oxygen (N1s/O1s) measured by XPS.

	PLA	$(PPG)_{10}$	AG73	AG73-G_3-$(PPG)_5$
N1s/C1s	0.01	0.07	0.09	0.18
N1s/O1s	0.00	0.03	0.04	0.09

3.3. Neurite outgrowth activity of peptide-adsorbed PLA films

The morphology of PC12 cells on peptide-adsorbed PLA films is shown in Fig. 3a, and the number of adhered PC12 cells with or without neurites is summarized in Fig. 3b. On the naked and $(PPG)_{10}$-adsorbed PLA films, PC12 cells (~230 cells mm^{-2}) did not adhere well enough, and so neurite outgrowth could not be found. This indicates that $(PPG)_n$ itself does not support bioactivity for cell adhesion or neurite outgrowth. Meanwhile, on AG73- and AG73-G_3-$(PPG)_5$-adsorbed PLA films, the adhesion of PC12 cells was improved to more than 300 cells mm^{-2}. Neurite outgrowth also occurred, i.e. the number of PC12 cells with neurites was more than 75% on both AG73- and AG73-G_3-$(PPG)_5$-adsorbed PLA films. In contrast, the mechanisms of adsorption of these films must be different from each other since the physicochemical properties of AG73 and AG73-G_3-$(PPG)_5$ are quite dissimilar. As mentioned above, they are considered to be adsorbed onto PLA films via

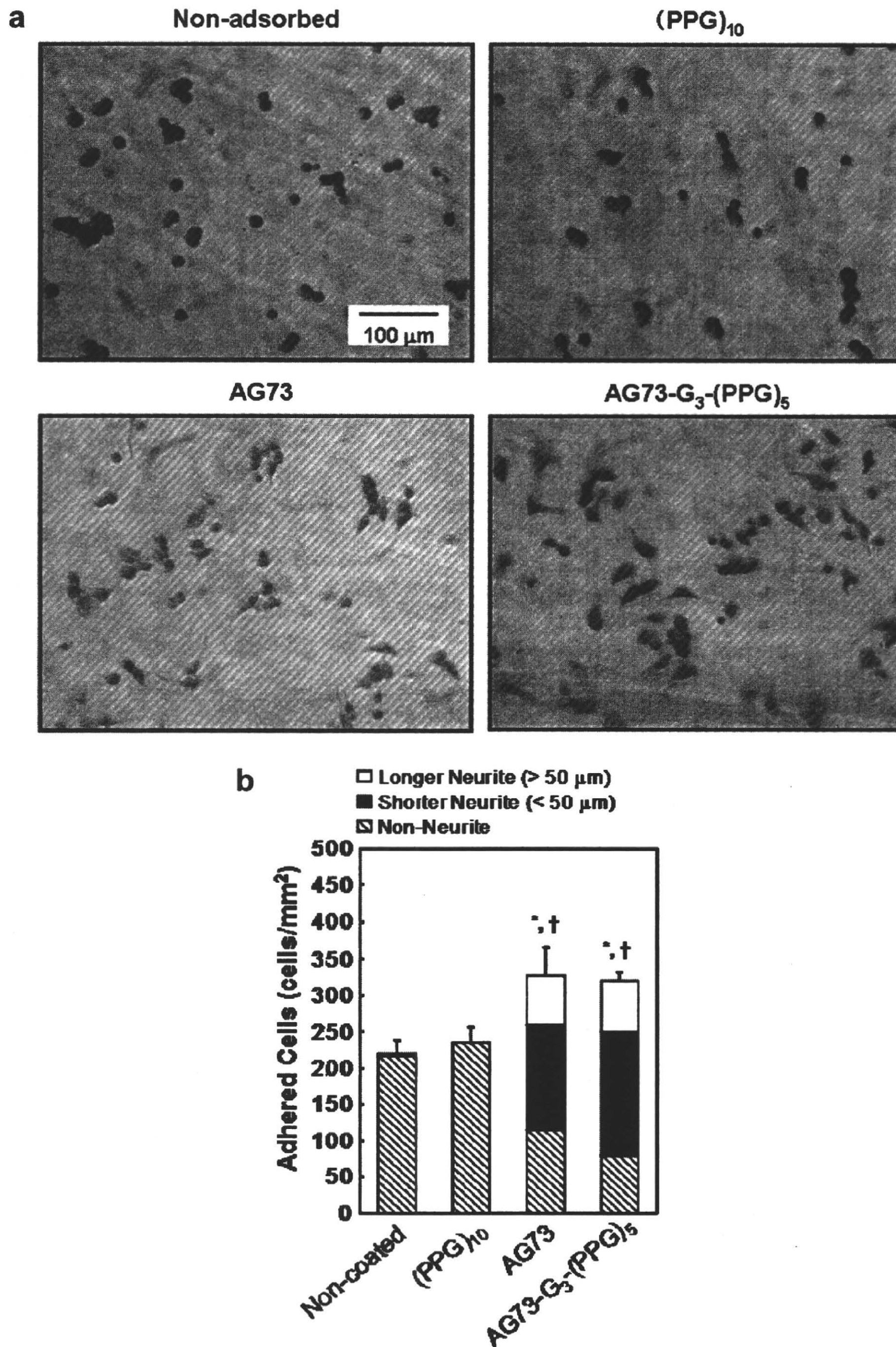

Fig. 3. Neurite outgrowth activity of PC12 cells on peptide-adsorbed PLA films. (a) Morphology of PC12 cells on peptide-adsorbed PLA films after 24 h. (b) The number of adhered PC12 cells on peptide-adsorbed PLA films with and without neurites. $^{*}P < 0.05$ when AG73 and AG73-G_3-$(PPG)_5$ groups compared with non-coated group; $^{\dagger}P < 0.05$ when AG73 and AG73-G_3-$(PPG)_5$ groups compared with $(PPG)_{10}$ group.

electrostatic and hydrophobic interactions, respectively. It is anticipated that under physiological conditions, adsorption of AG73-G_3-$(PPG)_5$ is more stable than that of AG73.

In order to evaluate the stability of the adsorbed AG73-G_3-$(PPG)_5$, neurite outgrowth assay was performed on AG73 and AG73-G_3-$(PPG)_5$-adsorbed PLA films after washing with 1.0 M NaCl aqueous solution (Fig. 4). In the case of AG73-adsorbed PLA films, the number of adhered PC12 cells is decreased to below 60% by 1.0 M NaCl washing, but the ratio of PC12 cells with neurites remains unchanged. After 1.0 M NaCl washing, the water contact angles of $(PPG)_{10}$-, AG73- and AG73-G_3-$(PPG)_5$-adsorbed PLA films changed from 70.1 ± 3.9°, 31.2 ± 2.1° and 55.6 ± 1.9° to 70.3 ± 1.5°, 60.0 ± 1.3° and 54.6 ± 4.2°, respectively. That is, the water contact angle of the AG73-adsorbed PLA films was drastically increased by 1.0 M NaCl washing, but that of AG73-G_3-$(PPG)_5$-adsorbed films was changed a little. These results indicated that adsorbed AG73 seemed to be partially removed by 1.0 M NaCl washing, and the remaining AG73 expressed neurite outgrowth-promoting activity, because AG73 was mainly adsorbed via electrostatic interaction. Meanwhile, the number of adhered PC12 cells was not changed and neurite outgrowth was also found on AG73-G_3-$(PPG)_5$-adsorbed PLA films after washing with H_2O or 1.0 M

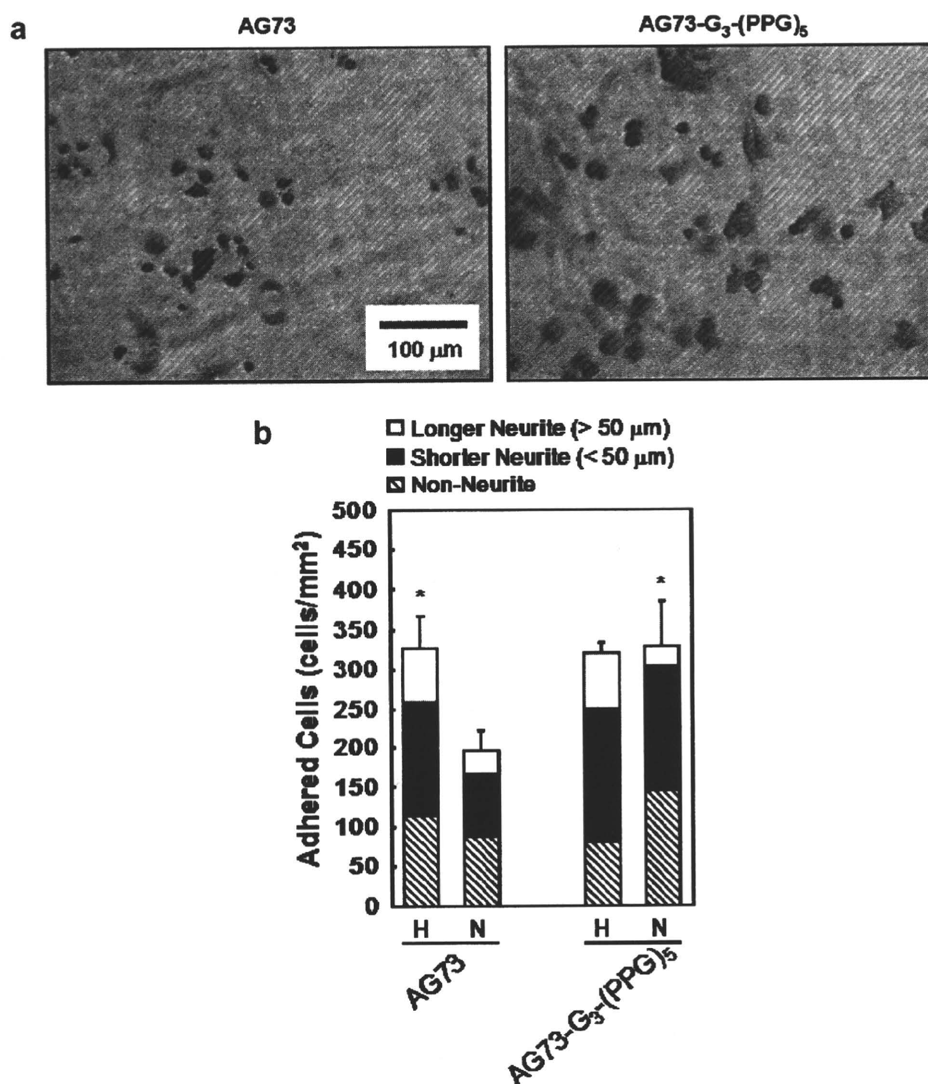

Fig. 4. Neurite outgrowth activity of PC12 cells on AG73- and AG73-G_3-$(PPG)_5$-adsorbed PLA films after washing with H_2O (H) or 1.0 M NaCl aq. (N). (a) Morphology of PC12 cells after 24 h. PLA films were modified with peptides, washed with 1.0 M NaCl and subjected to PC12 cell culture. (b) The number of adhered PC12 cells on peptide adsorbed PLA films with and without neurites. $^*P < 0.05$ when AG73 (H) and AG73-G_3-$(PPG)_5$ (N) groups are compared with AG73 (N) group.

NaCl. Hydrophobic interaction generally becomes stronger in the presence of salts because of the dehydration of the surface and adsorbents. The ratio of PC12 cells with neurites was slightly decreased by 1.0 M NaCl washing, because a partial AG73-G_3-$(PPG)_5$ was adsorbed by PLA films via electrostatic interaction. As result, it is proposed that PLA films adsorb AG73-G_3-$(PPG)_5$ mainly by hydrophobic interaction, and an ECM-like layer composed of structural protein and biosignalling sequences is formed. It is known that animal-derived collagen, like laminin, also promotes neurite outgrowth, because it is a fusion protein that combines a structural protein with many biosignal sequences [33]. Neurite outgrowth is mainly promoted by biosignal sequences in collagen, and it must be supported by the structural properties of these sequences. We believe that the structural properties of the ECM-like layer composed of AG73-G_3-$(PPG)_5$ creates a synergy with AG73 biosignalling for promoting neurite outgrowth.

4. Conclusion

Hydrophobic peptide-based interfacial adsorption onto PLA films and film stability have been characterized. Collagen-laminin mimics peptide AG73-G_3-$(PPG)_5$ and forms the hydrophobic PP-II structure in the $(PPG)_5$ region. Therefore, AG73-G_3-$(PPG)_5$ was capable of exhibiting stable adsorption onto PLA films via hydrophobic interaction, resulting in promotion of neurite outgrowth of PC12 cells. Furthermore, AG73-G_3-$(PPG)_5$, which is composed of biosignalling and structural protein-like sequences, forms an ECM-like layer on PLA films. It has recently been noted that the mechanical and morphological properties of ECMs are important for controlling stem cell differentiation [34]. The hydrophobic adsorption of collagen-like peptide is expected to serve as a surface modification technique of PLA films for controlling the biological properties of cells.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 3 and 4, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: 10.1016/j.actbio.2009.12.001.

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Peripheral Nerve Regeneration and Electrophysiological Recovery with CIP-Treated Allogeneic Acellular Nerves

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Abstract

Acellular nerve grafts are a desirable alternative to autografts, both because the source of acellular nerves is potentially unlimited and because they have the same matrix structure as natural nerves, which would facilitate axon growth from the defective nerve stump. Although some acellular nerves have been developed, most of them were studied in isogenic transplantation models and evaluated only by histological observation. In the present study, novel allogeneic acellular nerves prepared using the cold isostatic pressuring (CIP) method were developed and assessed as a potential substitute for autografts. The host immune response to acellular nerves and fresh nerves was analyzed using Lewis rats as donors and SD rats as recipients, which is the allogeneic transplantation model, by subcutaneous implantation for one month. In addition, sciatic nerve transplantation into a 10-mm nerve gap was carried out using the same model, and the axonal growth in acellular nerve transplantation was evaluated histologically and electrophysiologically, and compared with that of axons in the autograft transplant area. The subcutaneously implanted acellular nerves contained more macrophages and less vasculature than the allogeneic fresh nerves. In spite of these results of the subcutaneous implantation, Schwann cell infiltration in the graft transplanted into the sciatic nerve gap was observed after the short-term transplantation. The myogenic potential, which was measured as an index of electrophysiological function in acellular nerve transplantation, was also recovered in the long-term transplantation. Our results indicate that the acellular nerves developed herein have the potential to support nerve regeneration and might be useful as an alternative to autografts.

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Keywords

Acellular nerve, allogeneic, electrophysiological study, cold isostatic pressuring treatment

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