tion, and decreased sitosterol and cholesterol excretion into bile[26]. However, despite having normal plasma cholesterol levels, sitosterolemic patients often develop premature coronary heart disease (CHD), which suggests that high circulating levels of plant sterols per se might be atherogenic, even in non-sitosterolemic subjects. Glueck and coworkers were the first to address this issue, reporting that elevated serum concentrations of plant sterols in non-phytosterolemic subjects may be a risk factor for CHD[27]. In a study of 595 patients, they reported that slightly elevated plasma levels of plant sterols were a heritable maker of increased CHD risk. The Framingham Offspring Study also demonstrated that plasma levels of plant sterols, including sitosterol, campesterol and cholestanol, were significantly higher in patients with CHD (n=155) than in non-CHD controls (n=414)[28]. The most recent findings of the possibility of plant sterols being associated with an increased risk of CHD were derived from the Prospective Cardiovascular Münster (PROCAM) study[29]. Assmann *et al.* reported a nested case-control study of 159 cases with myocardial infarction and 318 matched controls from the PROCAM cohort. They found significantly higher serum concentrations of sitosterol in the cases as compared to controls (4.94±3.44 μmol/L vs. 4.27±2.38 μmol/L; p=0.028). They also demonstrated a 1.8-fold increase in the risk of CHD in subjects in the top quartile as compared with that in subjects with values in the lower three quartiles. In contrast, Wilund KR *et al.* demonstrated that ABCG5/G8-/- female mice fed western diets for 7 months against an LDL receptor knockout background exhibited no significant differences in the thoracic aorta lesion area as compared with littermate controls despite a 116-fold increase of the plasma sitosterol levels, and argued that there was no association between elevated plasma levels of plant sterols and atherosclerosis[30]. They also showed by analyzing data from the participants of the Dallas Heart Study that the mean and median levels of plasma sitosterol and campesterol were not significantly different between those with and without a family history of MI or premature CHD or between those with and without coronary calcium deposition as detected by electron beam computer tomography[30]. Thus, it remains under debate whether a modest increase of the serum plant sterol levels is a risk factor for CAD, although very high levels of plant sterols are known to be very atherogenic, as demonstrated in patients with sitosterolemia.

Current Therapy for Patients with Sitosterolemia

Dietary Therapy

A low plant sterol diet for patients with sitosterolemia should include the lowest amount of plant sterols possible. For such a diet, fruits, vegetables and cereal products without germ may be used, while vegetable fats and all plant foods with high fats, such as vegetable oils, margarine, nuts, avocados, and chocolate, should be avoided. Since the metabolism of shellfish sterols is also impaired in sitosterolemia, shellfish should also be avoided[31]. While dietary therapy has been shown to decrease serum levels of plant sterols in patients with sitosterolemia, in most cases, the levels do not reach the normal range. Also, some patients respond poorly to low-sterol diets.

Previous Pharmacologic Therapy

Resins

Bile acid binding resins are polymeric compounds that act as ion exchange resins. They substitute chloride anions with anionic bile acids and eventually interrupt the enterohepatic circulation of bile acids, consequently promoting bile acid excretion in the feces. Inhibition of the re-absorption of bile acids induces the stimulation of bile acid synthesis and concomitant reduction of intracellular cholesterol stores in hepatocytes, leading to a decrease in plasma LDL cholesterol levels by up-regulation of the LDL receptors. In most sitosterolemic patients, the serum levels of plant sterols also usually decrease along with the serum cholesterol level. Cholestyramine administration in addition to low-sterol diet therapy is effective for sitosterolemia. because this combination produces a significant reduction of the plasma levels of plant sterols, cholesterol, and cholestanol[32-35]. Cholestyramine treatment (12 g/day) in 4 homozygous sitosterolemic patients caused a 40 to 60% reduction of the serum levels of sitostanol, campestanol, sitosterol and campesterol in the plasma. In other studies, monotherapy with cholestyramine (8 to 15 g/day) or combined administration of cholestyramine with a low-sterol diet reduced plasma plant sterol concentrations by up to 50% in sitosterolemic patients. Nonetheless, not all patients respond similarly to cholestyramine treatment.

Statins

Statins are inhibitors of HMG-CoA reductase, which is a rate-limiting enzyme in the biosynthetic pathway of cholesterol, and have been widely used for decades for the treatment of hypercholesterolemia.

Patients with sitosterolemia exhibit abnormal regulation of the cholesterol synthetic pathway. They exhibit reduced cholesterol synthesis and reduced activities and expression levels of HMG-CoA reductase, in addition to paradoxically increased LDL-receptor activity, which is normally decreased in subjects with hypercholesterolemia. No mutations of the LDL receptor have been identified in patients with sitosterolemia investigated thus far. Actually, lovastatin and simvastatin failed to lower plasma sterol concentrations in patients with sitosterolemia. Thus, statins may have little beneficial effect on serum concentrations of sitosterol[34-36].

Sitostanol

Dietary sitostanol, which is a saturated sterol derived from sitosterol, has been well-documented to have a plasma cholesterol-lowering effect by decreasing cholesterol absorption; however, its effect on the serum levels of plant sterols in sitosterolemic patients is controversial. Lutjohann *et al.* noted a marked reduction of the serum levels of sitosterol and campesterol in two patients with sitosterolemia fed sitostanol for 4 weeks, and also reported that dietary sitostanol was not absorbed to any significant degree in these patients[37]. On the other hand, Connor *et al.* demonstrated that feeding margarine enriched with sitostanol and campestanol for 7–18 weeks to two patients with sitosterolemia decreased their plasma cholesterol, campesterol, and sitosterol levels, but increased their plasma sitostanol and campestanol levels. An animal study from the same report showed that plasma plant stanol levels were elevated and the absorbed plant stanols were deposited in rat tissues after the animals were fed high-cholesterol diets supplemented with sitostanol for 4 weeks, raising a warning against treatment of sitosterolemic patients with plant stanols[38].

Surgical Therapy (Partial Ileal Bypass Surgery)

Increased intestinal bile acid loss can also be induced by partial ileal bypass surgery, which is defined as shortening of the ileum to reduce the total small intestinal length. In subjects with sitosterolemia subjected to partial ileal bypass surgery, considerable reduction of the total plasma levels of sterols was achieved[24]; plant sterol and 5α-saturated stanol concentrations declined by 55% in two patients, and a similar degree of decrease of plasma apolipoprotein B levels was also observed. Reduction of the plasma sterol concentrations was associated with clinical improvement, including the disappearance of aortic systolic murmurs. The observations that cholesterol synthesis and microsomal HMG-CoA reductase activity were not stimulated in mononuclear leukocytes isolated from sitosterolemia patients after ileal bypass surgery or cholestyramine treatment have been interpreted as supporting the hypothesis of an inherent defect in cholesterol synthesis in patients with sitosterolemia[39, 40].

Plasmapheresis

It has been suggested that plasmapheresis might be effective for the treatment of sitosterolemia; however, until date, no results of well-organized trials have been reported.

Novel Strategy for the Treatment of Sitosterolemia with Ezetimibe

The above described therapies have only been partially effective in patients with sitosterolemia. Low-sterol diets are effective in some cases[33]; however, other reports have shown that diet therapy remains difficult to comply with, is often unsuccessful for lowering plasma levels of plant sterols, and has been shown to produce adversely contradictory increases in plasma plant sterol levels[34, 41]. While bile salt-binding resins may lower plant sterol levels, they do not normalize them. Furthermore, no decline of plasma sterol levels is noted in patients treated with statins. Thus, there is an imperative need to develop novel therapies for the optimal treatment of sitosterolemia.

Ezetimibe is a lipid-lowering drug that inhibits the absorption of dietary and biliary cholesterol. Meta-analysis of 8 randomized, double-blind, placebo-controlled trials revealed that ezetimibe monotherapy for 12 weeks for the treatment of primary hypercholesterolemia was associated with a statistically significant reduction of the mean plasma LDL cholesterol levels by 18.58% as compared with a placebo[42]. Altmann and coworkers revealed Niemann-Pick C1-like 1 (NPC1L1) as a strong candidate for the ezetimibe-sensitive cholesterol transporter[43, 44]. NPC1L1 protein has ~50% amino acid sequence homology to NPC1, which is defective in the cholesterol storage disease Niemann-Pick type C, and functions in intracellular cholesterol trafficking. In contrast to NPC1, NPC1L1 is expressed predominantly in the liver and small intestines, with peak expression in the proximal jejunum, in parallel with the efficiency of cholesterol absorption along the gastrocolic axis. Subfractionation of the brush border membrane suggested that NPC1L1 is associated with the apical membrane fraction of enterocytes. NPC1L1-null mice substantially reduced the intestinal uptake of cholesterol and sitosterol with markedly reduced plasma phytosterol levels. Ezetimibe was demonstrated to bind directly to

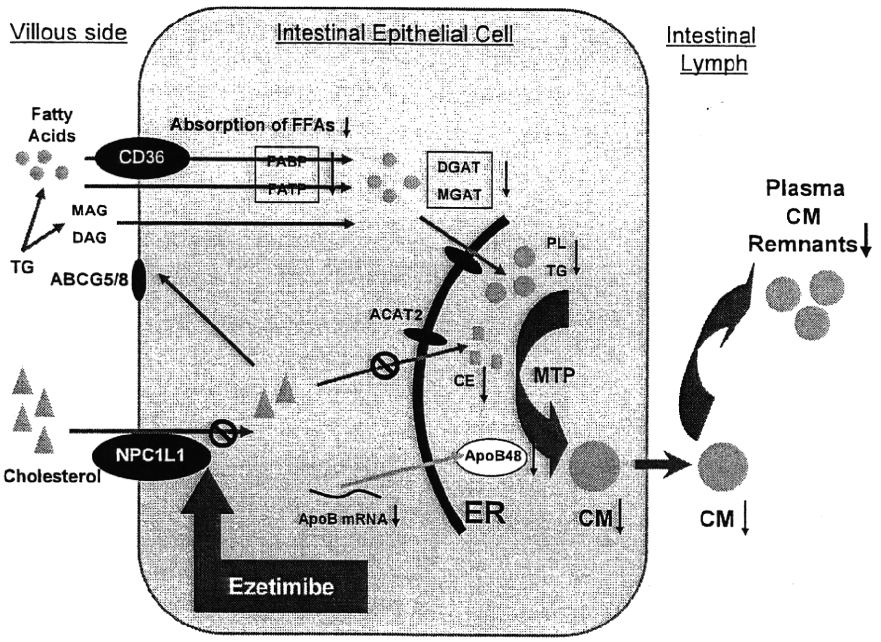

Fig. 2. Molecular mechanisms for the effect of ezetimibe on cholesterol and plant sterol absorption.

CE: Cholesteryl Esters, CM: Chylomicrons, FFA: Free Fatty Acid, TG: Triglycerides, MAG: Monoacylglycerol, DAG: Diacylglycerol, MGAT: Monoacylglycerol acyltransferase, DGAT: Diacylglycerol acyltransferase, MTP: Microsomal triglyceride transfer protein, ACAT2: Acyl-CoA: cholesterol acyltransferase-2, FABP: Fatty acid binding protein, FATP: Fatty acid transport protein.

NPC1L1 and act by blocking sterol-induced internalization of NPC1L1, subsequently inhibiting the absorption of sitosterol as well as cholesterol (**Fig. 2**) Indeed, sitosterol and campesterol were reported to be reduced by 41% and 48%, respectively, after 2 weeks of ezetimibe administration in 18 patients with mild to moderate hypercholesterolemia[45]. Because of these action mechanisms, ezetimibe has the potential to be a revolutionary treatment agent for sitosterolemia. Salen and coworkers reported a multicenter, double-blind, randomized, placebo-controlled study in which they showed the augmented effect of ezetimibe in 37 patients with sitosterolemia[46]. They showed that serum sitosterol concentrations were reduced by 21% in patients treated with ezetimibe 10mg/day (n=30), as compared with a non-significant 4% rise in the placebo group (n=7), with no serious treatment-related adverse events or discontinuation due to the development of adverse events.

Here, we present 2 Japanese adolescent cases of sitosterolemia, one underwent percutaneous coronary intervention for accelerated coronary arterial atherosclerosis, and plasma sitosterol levels were successfully reduced by ezetimibe.

Case 1

A 23-year-old female patient had been diagnosed as having sitosterolemia at the age of 13. Her serum sitosterol level was 243 μg/mL and total cholesterol level was 458 mg/dL at diagnosis. She had had a history of multiple xanthelasmas, xanthomas on the knee joints and buttocks, and a marked thickening of the Achilles tendon since the age of 3. Genetic analysis revealed point-mutations in the ABCG5 gene (**Table 1**). She had been treated with probucol, cholestyramine and colestimide since she was 9 years old, and while her serum total cholesterol level had declined to the normal range (172 mg/dL), serum sitosterol levels remained very high.

In 2004, when she was 18 years old, she developed frequent episodes of anterior chest pain on exertion and coronary angiography confirmed severe stenosis of the right coronary artery (**Fig. 3**). Percutaneous coronary intervention (PCI) was successfully performed and the angina symptoms resolved. However, serum sitosterol levels were still markedly elevated (87.8 μg/mL), underscoring the urgent need to lower plant sterol levels so as to prevent further progression

Table 1. Location of the gene mutation and hematological/biochemical laboratory data in case 1 (at the time of PCI) and case 2 (at the first visit)

	Case 1	Case 2
ABCG5 mutation	A1756C (R550S) G1362A (R419H)	G1306A (R389H) C1949 (Q604E)
WBC (/μL)	7,610	6,370
RBC ($\times 10^4$/μL)	382	385
Hemoglobin (g/dL)	9.8	10.9
Platelet ($\times 10^4$/μL)	18.5	12.5
AST (IU/L)	14	29
ALT (IU/L)	16	26
CRP (mg/dL)	0.3	1.0
Total Cholesterol (mg/dL)	172	289
LDL Cholesterol (mg/dL)	87	229
HDL Cholesterol (mg/dL)	67	85
Triglyceride (mg/dL)	121	172
Sitosterol (μg/mL)	87.8	88.5
Campesterol (μg/mL)	48.8	84.5
Stigmasterol (μg/mL)	4.0	7.2

of atherosclerosis. As ezetimibe was not approved in Japan at that time, we succeeded in obtaining the approval of the Ethics Committee of Osaka University Hospital. We then initiated ezetimibe (10 mg/day) in January 2006. As shown in **Table 2**, her serum sitosterol level decreased markedly following the start of ezetimibe treatment (51.3% reduction as compared to pretreatment), with no apparent development of any adverse effects. Until the present, more than 3 years after PCI, the patient had remained free of cardiovascular symptoms, with persistently reduced serum plant sterol levels (**Fig. 4**).

Case 2

An 18 year-old male subject had been diagnosed with hypercholesterolemia (total cholesterol: 353 mg/dL) when he was 8 years old. Treatment with colestimide, simvastatin and rosuvastatin failed to reduce the plasma cholesterol levels. He was therefore referred to Osaka University Hospital, where he was diagnosed as having sitosterolemia at the age of 16 years with point mutation of ABCG5, and a serum sitosterol level of 88.5 μg/mL (**Table 1**). He exhibited subclinical progression of atherosclerosis, as assessed by carotid ultrasound examination, which revealed a mild atherosclerotic plaque in the right common carotid artery, which was unusual for his age. He was then prescribed ezetimibe (10 mg/day) in addition to rosuvastatin and colestimide from July 2006, which led to a 48.9% reduction in the serum sitosterol level (**Fig. 5**).

Better reduction of the plasma sterol levels have been achieved in these two patients with ezetimibe

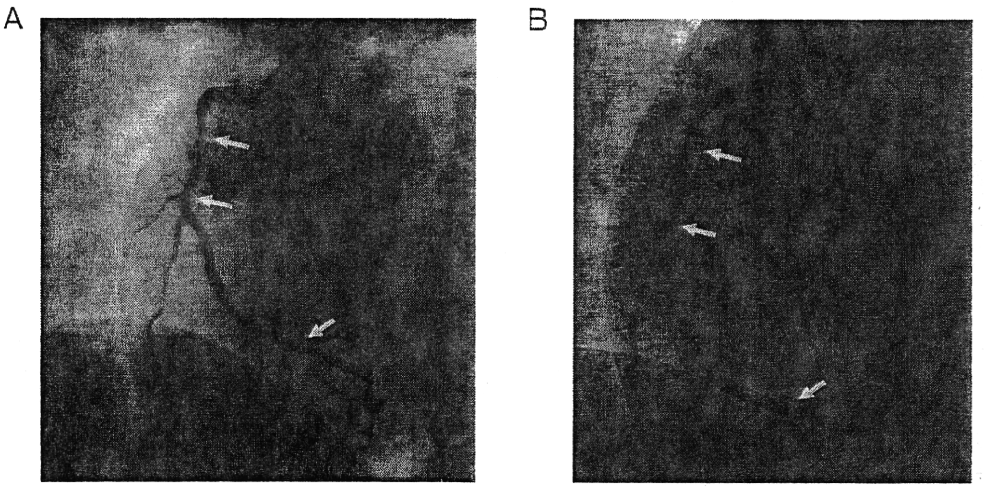

Fig. 3. Coronary angiography in case 1.

A. Left anterior oblique (LAO) view of the right coronary artery before PCI. Moderate to severe atherosclerotic stenotic lesions are observed in the proximal to distal right coronary artery (arrows). The left coronary artery was not affected.

B. LAO view of the right coronary artery after PCI. Coronary stents were successfully implanted for proximal diseased lesions and plain balloon angioplasty was performed for the distal lesion. (Figures reproduced and reprinted with permission from Koseki M, Mitsusada N, Yamashita S. The Lipids Vol19 No.4: 421-424, 2008. Copyright 2008. p422 Medical Review Co. Ltd)

Table 2. Serum sterol concentrations before and after ezetimibe treatment

	before	after	% reduction
Case 1			
Total Cholesterol (mg/dL)	198.4 ± 60.5	174.2 ± 14.2	12.2
Sitosterol (μg/mL)	88.7 ± 8.1	43.2 ± 7.2	51.3
Campesterol (μg/mL)	48.8 ± 17.3	35.0 ± 5.0	28.3
Stigmasterol (μg/mL)	4.0 ± 0.2	2.9 ± 0.3	27.5
Case 2			
Total Cholesterol (mg/dL)	179.5	136.0 ± 16.3	24.2
Sitosterol (μg/mL)	87.8	44.9 ± 8.5	48.9
Campesterol (μg/mL)	75.4	37.4 ± 4.2	50.4
Stigmasterol (μg/mL)	4.4	2.6 ± 0.4	40.9

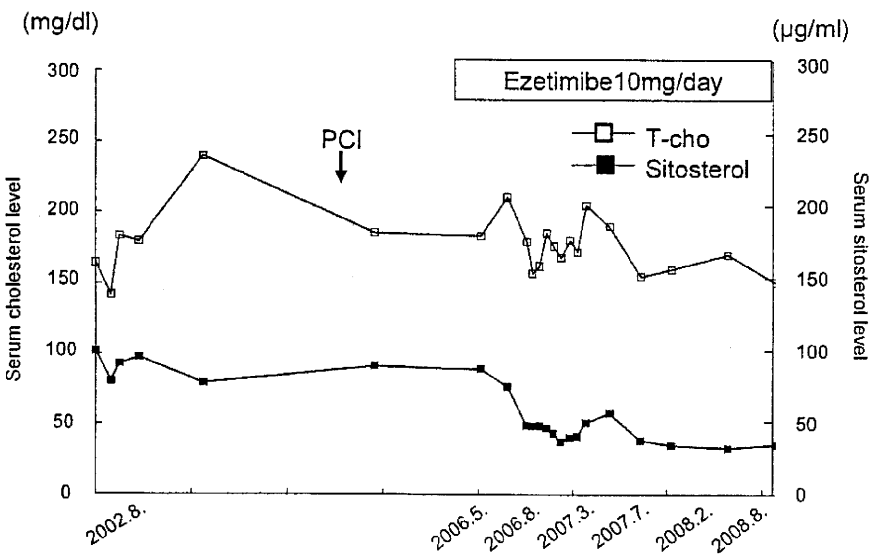

Fig. 4. Changes in serum sterol levels in case 1 after ezetimibe treatment.

Open and filled squares indicate serum total cholesterol (mg/dL) and beta sitosterol (μg/mL) levels, respectively. Upper panel shows the history of prescribed medications. The arrow indicates the time-point at which case 1 underwent PCI.

treatment as compared with previous reports, and the reduction of plant sterol levels has been sustained since the start of therapy. It is crucial to observe the course of atherosclerosis progression in these patients in the future. At the time of writing, they have been treated with ezetimibe for more than 3 years and no obvious adverse effects have been detected.

Recently, the ENHANCE trial reported that in patients with heterozygous familial hypercholesterolemia, combined ezetimibe + simvastatin therapy did not result in any significant change of the intima-media thickness as compared with simvastatin therapy alone, in spite of decreasing the plasma LDL cholesterol and C-reactive protein levels[47]. More recently, the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial revealed that combined therapy with simvastatin + ezetimibe resulted in a significant reduction of the risk of ischemic cardiovascular events, mainly fewer CABG procedures. However, no significant difference was detected in the secondary outcome of aortic-valve-related events between the simvastatin + ezetimibe and control groups[48].

Otherwise, the results of the SEAS trial highlighted the growing debate of whether ezetimibe administration in addition to a statin to achieve more marked lowering of the serum LDL cholesterol level

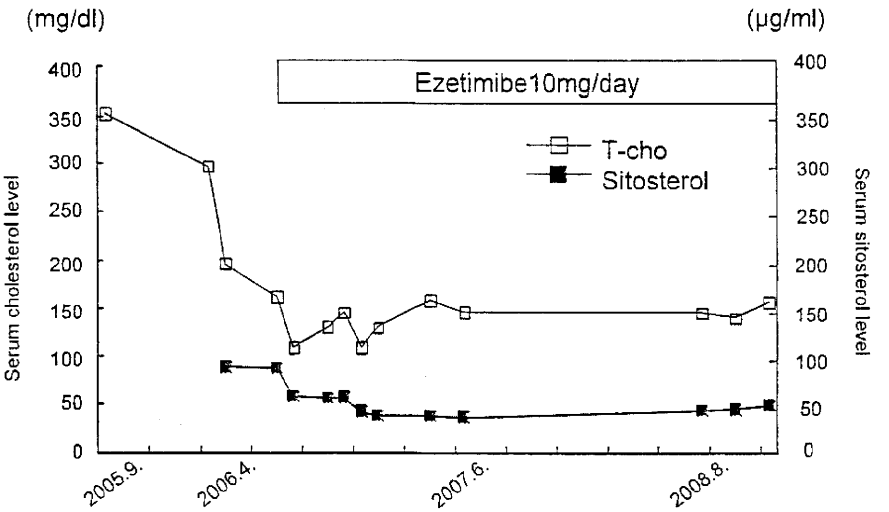

Fig. 5. Changes in serum sterol levels in case 2 after ezetimibe treatment.

Open and filled circles indicate serum total cholesterol (mg/dL) and beta sitosterol (μg/mL) levels, respectively. Upper panel shows the history of prescribed medications.

might increase the incidence of cancer. Peto R analyzed the incidence of cancer in the subjects of the SEAS trial (mean follow-up, 4.1 years) with those of two currently ongoing large trials, the Study of Heart and Renal Protection (SHARP) (mean follow-up, 2.7 years) and the Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) (mean follow-up, 1.0 year). They concluded that the available results from these three trials did not provide any credible evidence of an adverse effect of ezetimibe on the incidence rate of cancer[48, 49]. Non-clinical data obtained from chronic administration studies in 3 species and 2-year carcinogenicity studies in rats and mice conducted by Halleck M also suggest that ezetimibe is not carcinogenic[50]. Nonetheless, longer follow-up is necessary to build a consensus on the long-term safety and efficacy of ezetimibe in the prevention of cardiovascular diseases.

Conclusion

Taken together, ezetimibe is a novel and potent treatment agent for patients with sitosterolemia and could work additively with conventional therapy. The long-term efficacy for cardiovascular event reduction and the safety of ezetimibe in both hypercholesterolemic and sitosterolemic patients will provide further insight into the atherogenicity of plant sterols.

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Original Article

HDL/Apolipoprotein A-I Binds to Macrophage-Derived Progranulin and Suppresses its Conversion into Proinflammatory Granulins

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Aim: HDL has anti-inflammatory effects on macrophages, although the mechanism of action remains unclear. We hypothesized that HDL suppresses the conversion of macrophage-secreted factors into proinflammatory factors via binding, and tried to identify the factor that could form a complex with HDL and/or apolipoprotein (apo) A-I.

Methods and Results: In conditioned media obtained from human monocyte-derived macrophages, we found an apo A-I binding protein and identified the protein as progranulin/proepithelin/acrogranin/PCDGF. Co-immunoprecipitation analysis showing that progranulin binds and forms a complex with apo A-I and the presence of progranulin in the HDL fraction in the sera indicated that progranilin is a novel apolipoprotein. Conditioned media of HEK293 cells transfected with progranulin augmented the expression of TNF-alpha and IL-1-beta on macrophages, but these effects of progranulin were inhibited by co-incubation with HDL or apo A-I. Anti-progranulin antibodies also reduced the expression of TNF-alpha and IL-1-beta on macrophages. Granulins as conversion products derived from progranilin increased TNF-alpha and IL-1-beta expression and the effects were not suppressed by HDL.

Conclusions: Our results suggest that the anti-inflammatory effects of HDL on macrophages might be due to suppression of the conversion of progranulin into proinflammatory granulins by forming a complex.

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Key words; HDL, Apolipoprotein A-I, Progranulin, Proepithelin, Acrogranin, PCDGF, Macrophage

Introduction

Several pathological studies have shown that low high-density lipoprotein (HDL) levels are associated with plaque instability in patients with acute coronary syndrome[1]. Accordingly, the reverse cholesterol transport system, in which excess cholesterol is extracted from atheromatous plaques, is considered important in stabilizing plaques and preventing plaque rupture[2, 3]. A recent study reported that in addition to the reverse cholesterol system, HDL infusion could stabilize atheromatous plaque through its anti-inflammatory properties[4].

The acute phase of a coronary event is associated with a significant fall in serum levels of apolipoprotein (apo) A-I (a major component of HDL) and HDL-C[5]. There is evidence that plasma HDL-C measured in the initial stage of the acute phase of coronary events predicts the risk of recurrent cardiovascular events over the ensuing 16 weeks[6]. We often expe-

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rience patients with acute coronary syndrome whose HDL levels are reduced before the occurrence of plaque instability[4].

Infiltration and accumulation of foam cells (macrophages) is a characteristic feature of atheromatous plaques[7]. Once activated, macrophages secrete various pro-inflammatory cytokines and proteases, which could result in plaque instability and rupture[7]. Are the anti-inflammatory effects of HDL mediated through suppression of the secretion of such cytokines from macrophages? The main theme of our research is to determine the mechanisms underlying the reduction of serum HDL during the acute phase of coronary events. In this study, we hypothesized that HDL modulates the expression levels of pro-inflammatory cytokines secreted by macrophages. Progranulin is here described as a macrophage-derived secretory factor, which is a pluripotent protein and a precursor of its proteolytic peptides, granulins, whose functional properties were different from their intact precursor in some cases[8], and whose pro-inflammatory properties were suppressed via binding to HDL.

Materials and Methods

Lipoprotein Isolation

Apo A-I was purchased from Sigma Aldrich (St. Louis, MO). HDL3 were isolated from human serum by ultracentrifugation at a density of 1.125-1.210 g/mL[9].

Isolation of Human Monocyte-Derived Macrophages

Mononuclear cells were isolated from the buffy coats of plasma collected from healthy volunteers using density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). The cells were then cultured for 7 days, as described previously[9].

Preparation of Conditioned Medium

A monolayer of macrophages was collected from 7-day culture and incubated in serum-free RPMI1640 for 24 h at 37℃. The conditioned medium was collected and replaced with fresh RPMI1640 every 24 hours for 5 days. The collected medium was centrifuged, and the supernatant was treated with benzamidine hydrochloride (Sigma) at a final concentration of 1 mM to protect against protease degradation.

Ligand Blotting Analysis

The concentrated conditioned medium was separated under non-reducing conditions using 10-20% polyacrylamide gradient gels, transferred onto nitrocellulose membranes, and blotted with 5 μg/mL biotinylated-apo A-I. After incubation with peroxidase-conjugated streptavidin, the blots were visualized with an ECL kit (Amersham Pharmacia Bioscience, Uppsala, Sweden).

Purification of Apo A-I Binding Protein from Macrophage-Conditioned Medium

The conditioned medium (total volume, 20 L) was collected and then treated with 80% ammonium sulfate. The precipitate was dissolved in 2.5 mL, and then desalted and equilibrated into Tris-buffered saline (20 mmol/L Tris HCl, pH 7.4, and 135 mmol/L NaCl) using a PD-10 column (Amersham Pharmacia Biotech). The eluate was added to an apolipoprotein A-I-affinity column (Amino Link; Amersham Pharmacia Biotech), and allowed to stand overnight at 4℃. After vigorous washing with Tris buffer with 1 mol/L NaCl (20 mmol/L Tris/HCl, pH 7.4, and 1 mol/L NaCl), the binding proteins were eluted with 8 mol/L urea. The eluate was concentrated with Amicon Ultra-15 50,000 MWCO (Millipore, Bedford, MA) to ensure purity, and subjected to SDS-PAGE.

Amino Acid Sequencing

The purified apo A-I-binding protein was applied for in-gel digestion with V8 endopeptidase, transferred to a polyvinylidene fluoride (PVDF) membrane, and stained with Coomassie brilliant blue (CBB) R-250. The three apparent fragmented bands were subjected to amino acid sequencing in a sequencer (Perkin Elmer-Cetus, Foster City, CA).

Construction of Progranulin, Granulin A and Granulin B Expression Vector

The expression vectors of progranulin (aa 1-593, see RESULTS), myc-His-tagged progranulin and granulin were constructed from pcDNA3.1, as described previously[10]. In short, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5'-aggaccgcggagtcggacgcaggcagacca-3' and 5'-tccgagtggggtcccgagggctgcagagtc-3', and for nested PCR, primer pairs 5'-gtcggactccggcagaccatgtggaccctg-3' and 5'-agggctcgagagtcttcagtactgtcccte-3'. The nested PCR product was digested with Bam HI and Xho I and the digested product was ligated into pcDNA3.1 pretreated with Bam HI and Xho I. The construct was used as a progranulin expression vector. To obtain myc-His tagged progranulin expression vector, cDNA obtained from human monocyte-derived macrophages underwent PCR using primer pairs 5'-aggaccgcggagtcggacgcaggcagacca-3' and 5'-tccgagtggggtcccgagggctgcagagtc-3', and for nested PCR, primer pairs 5'-gtcggactccggcagaccatgtggaccctg-3' and 5'- tccc-

tcacctctagagcagctgtctcaagg-3', and the nested PCR product was digested with Bam HI and Xba I and the digested product was ligated into pcDNA3.1/myc-His vector pretreated with Bam HI and Xba I. Human granulin A (aa 281–337) and B (aa 206–261) vectors were constructed with secretion being driven by the human progranulin signal peptide (aa 1–17).

Immunoprecipitation

The vectors were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp., Carlsbad, CA, USA). Three days after transfection, HEK293 cells were incubated with conditioned medium containing apo A-I (final concentration 5 μg/mL) at 37℃ for 30 min. The apo A-I-containing media were collected and immunoprecipitated with anti-progranulin antibody (clone N19; Santa Cruz Biotechnology, Santa Cruz, CA), pulled-down with protein G (Amersham Pharmacia Biotech), separated by reducing SDS-PAGE, and Western blotted with anti-apo A-I antibody, or the reverse. *In vitro* translated progranulin was produced using an *in vitro* translation system (Duo, Tokyo, Japan), and mixed with apo A-I at a final concentration of 5 μg/mL. The mixture was then subjected to co-immunoprecipitation analysis.

Immunoblotting Analysis

The conditioned medium was subjected to immunoblotting analysis with anti-progranulin monoclonal antibody (clone N-19; Santa Cruz Biotechnology). The blots were visualized after incubation with peroxidase-conjugated anti-mouse IgG antibody (DAKO, Denmark).

Quantitative Real-Time PCR

The constructed expression vectors of progranulin, granulin A and granulin B, were transiently expressed in HEK293 cells using a Calcium Phosphate Transfection Kit (Invitrogen Corp.). Three days after transfection, the conditioned media were obtained and macrophages were incubated in conditioned media with or without HDL (10 μg/mL) or apo A-I (5 μg/mL) for 24 h. Total RNA was then isolated using RNeasy MINI kits (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. For cDNA synthesis, 600 ng total RNA was reverse transcribed using SuperScript Ⅲ RTase (Invitrogen, San Diego, CA). TaqMan probe and primers for progranulin, CD14, CD36, CD68, TNF-alpha, IL-1beta and GAPDH were purchased from Applied Biosystems (Assay ID: Hs00173570_m1, Hs02621496_S1, Hs01567186_m1, Hs00154355_m1, Hs99999043_m1, Hs99999029_m1 and Hs00266705_g1, respectively). Quantitative real-time PCR was performed using the ABI Prism 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). The cDNA samples (10 ng in a total volume of 10 μL) were mixed with primers, probe and TaqMan Universal PCR Master Mix as described in the accompanying sheet supplied by the manufacturer (Applied Biosystems). PCR was conducted using the following settings: 50℃ for 2 min, 95℃ for 10 min and 40 cycles at 95℃ for 15 s and 60℃ for 1 min.

Results

Purification of Progranulin as Apo A-I Binding Protein

We first tested whether HDL suppresses the pro-inflammatory cytokines secreted by macrophages by forming complexes with them, and thus searched for hypothetical proteins. The conditioned media obtained from 7-day-cultured human macrophages were concentrated with the ammonium sulfate precipitation method, desalted and subjected to ligand blotting analysis using biotinylated-apo A-I as a ligand. After separating the media on SDS-PAGE under non-reducing conditions, we detected an apo A-I-binding protein with a MW of 130 kDa (**Fig. 1A**). To purify the protein, the concentrated medium was subjected to an affinity column with immobilized human apo A-I and we obtained a concentrated eluate. To ensure purity, 10 μg proteins of the conditioned medium and 0.5 μg of the eluted protein were subjected to SDS-PAGE. Silver staining showed a single band of 80 kDa protein (**Fig. 1B**).

In the next step, we obtained three polypeptide fragments after in-gel-digestion with V8 proteases. Next, the amino-terminal sequence of these polypeptides was determined. The amino acid sequences were "avacgdgh", "nattdllt" and "kapahlsl", respectively. All amino acid sequences were identical to those of progranulin (aa 89–96, 265–272 and 347–354, respectively).

To confirm that progranulin could bind to HDL, HDL fractions (0.5 μg) were applied for immunobloting with anti-progranulin antibody (clone N19). As shown in **Fig. 1C**, progranulin was observed in the HDL fraction, and a small amount was observed in serum. As indicated in **Fig. 1A**, HDL binding protein had approximately 130kDa molecular weight in non-reducing conditions; however, MW of progranulin was approximately 80 kDa, as shown in **Fig. 1B**. To clarfy these discrepancies, purified proteins by affinity column with immobilized human apo A-I were

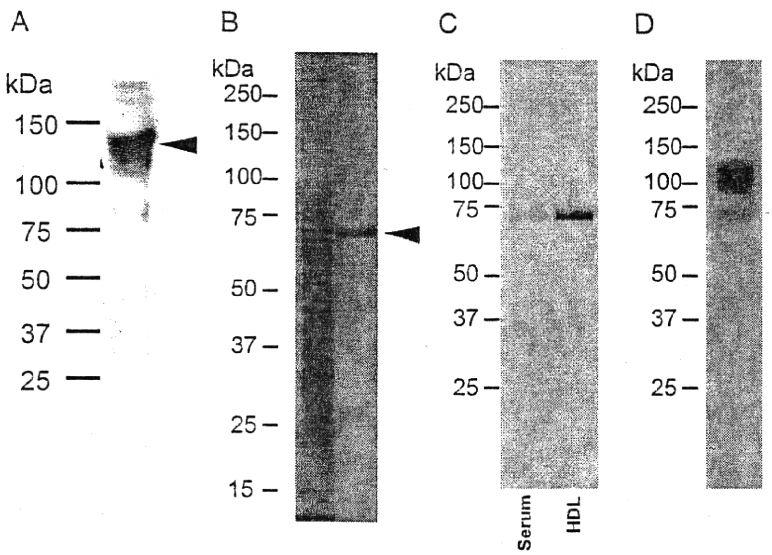

Fig. 1. Purification of apolipoprotein A-I binding protein from macrophage-conditioned medium.

(A) SDS-PAGE of purified apo A-I binding protein from macrophage-conditioned medium. The conditioned medium derived from human macrophages (150 μg/lane) was subjected to 4/20% gradient SDS-PAGE under non-reducing conditions, and subjected to ligand blotting analyses with 5 μg/mL biotinylated apo A-I.
(B) Ligand blotting of the purified apo A-I binding protein in macrophage-conditioned medium. The conditioned medium derived from macrophages was collected, concentrated, and then 10 μg proteins of the conditioned medium were subjected to SDS-PAGE and silver staining (left lane). After purification of apo A-I binding protein, 0.5 μg of the purified protein was used for SDS-PAGE and silver stained to ensure purity (right lane).
(C) Immunoblotting of HDL fraction with anti-progranulin. Human serum and HDL fractions (0.1 μg) were applied for immunobloting with anti-progranulin antibody (clone N19).
(D) Progranulin could form a homo-dimer. Proteins purified by affinity column with immobilized human apo A-I were applied for imunoblotting under non-reducing conditions. Progranulin-like immunoreactive bands were observed in 130kDa and 80 kDa.

applied for imunoblotting in non-reducing conditions. Progranulin-like immunoreactive bands was observed in 130 kDa and 80 kDa (**Fig. 1D**), indicating that progranulin could form a homo-dimer.

Formation of Progranulin-Apo A-I Complex

To confirm the formation of progranulin-apo A-I complex, we performed co-immunoprecipitation analysis (**Fig. 2**). Progranulin-expressing conditioned medium treated with apo A-I was subjected immunoprecipitated with anti-apo A-I antibody to bring down apo A-I and then Western blotted with anti-progranulin antibody or the reverse (**Fig. 2A, B**). The two proteins were recovered simultaneously, indicating that progranulin binds apo A-I. To further confirm the formation of the protein complex, progranulin was translated *in vitro*, co-incubated with apo A-I and then subjected to co-immunoprecipitation analysis (**Fig. 2C, D**). Progranulin, *in vitro* translated under non-reducing conditions (**Fig. 2C, D**), was detected in apo A-I immunoprecipitates while apo A-I was identified in progranulin immunoprecipitates or the reverse. These results indicate that progranulin and apo A-I could bind each other.

Progranulin-Expressing Macrophages

Although we purified and identified progranulin as an apo A-I binding protein from conditioned media derived from macrophages, it is important to confirm that macrophages express and produce progranulin. As shown in **Fig. 3A**, macrophages expressed progranulin and the expression level was dependent on mac-

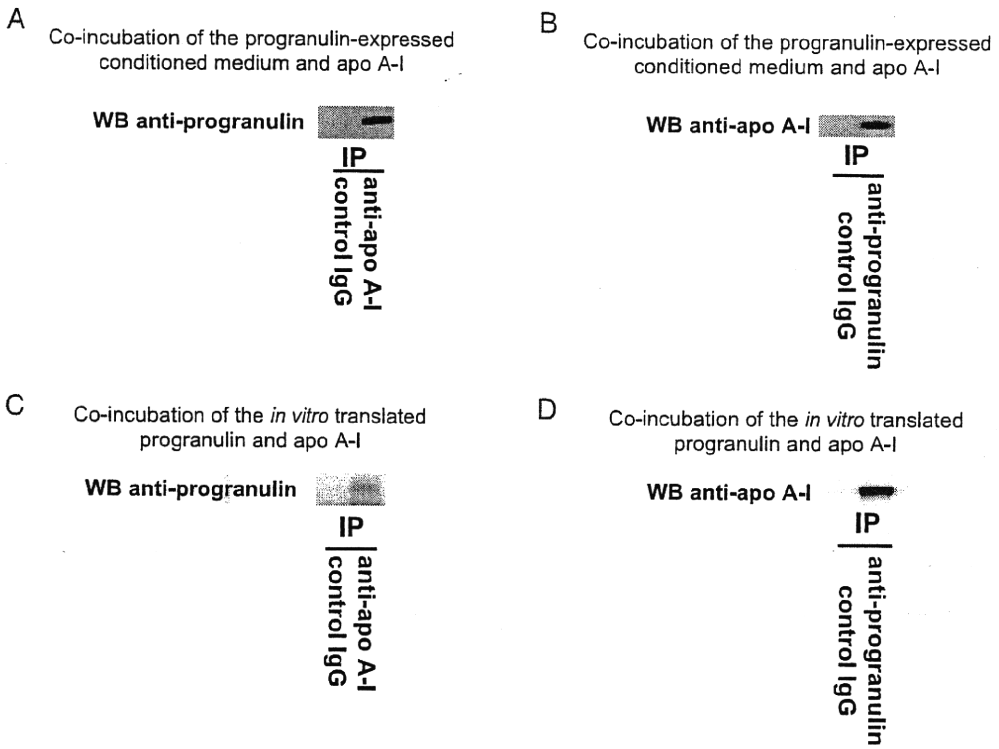

Fig. 2. Progranulin and apolipoprotein A-I form a complex.

(A) Formation of a protein complex from progranulin, transfected and secreted from HEK293 cells, and apolipoprotein A-I. The conditioned medium of HEK293 cells, transfected with pcDNA3.1 expression vector of progranulin, and apo A-I, was immunoprecipitated using rabbit anti-apo A-I antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-progranulin antibody, and then visualized.

(B) Formation of a protein complex from progranulin, transfected and secreted from HEK293 cells, and apolipoprotein A-I. The conditioned medium of HEK293 cells, transfected with pcDNA3.1 expression vector of progranulin, and apo A-I, was immunoprecipitated using rabbit anti-progranulin antibody. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized.

(C) Progranulin, translated under native but not reducing conditions, and apolipoprotein A-I form a complex. The *in vitro* translated progranulin and apo A-I were immunoprecipitated using rabbit anti-apo A-I antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-progranulin antibody (N19), and then visualized.

(D) Progranulin, translated under native but not reducing conditions, and apolipoprotein A-I form a complex. The *in vitro* translated progranulin and apo A-I were immunoprecipitated using rabbit anti-progranulin antibody. Similar samples were immunoprecipitated using control rabbit IgG as a negative control. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized.

rophage differentiation; gene expression was higher after 5-day culture. Next, we examined progranulin protein production by macrophages (**Fig. 3B**). After cultivation for the indicated time, macrophages were incubated without serum for 24 h and their conditioned media were blotted with anti-progranulin antibody. The production of progranulin protein by macrophages increased in a macrophage differentiation-dependent manner, similar to the gene expression. To examine whether progranulin could augment the expression of progranulin itself, TNF-alpha and IL-1-beta, 7-day-cultured human macrophages were cultured for 24 hours with progranulin. As shown in **Fig. 3C**, progranulin increased the expression of pro-

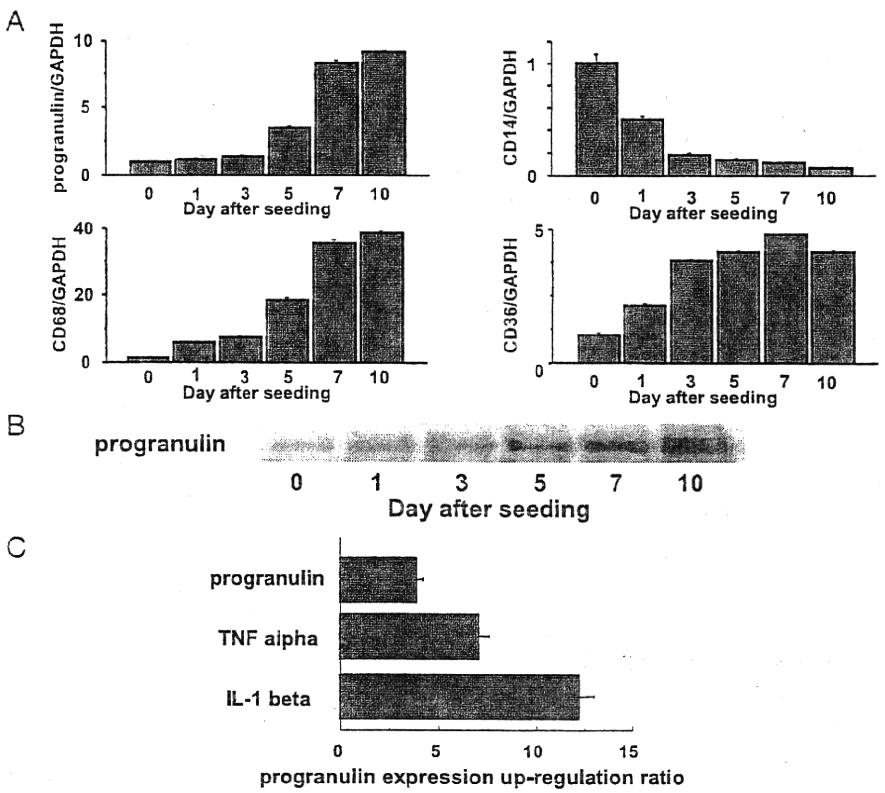

Fig. 3. Human monocyte-derived macrophages express and secrete progranulin into conditioned media.

(A) Expression of progranulin on macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages expressed progranulin and the expression level was dependent on macrophage differentiation by quantitative PCR. Maturation of monocytes-macrophages was associated with over-expression of CD36 and CD68 and under-expression of CD14. Data are the mean ± SE of 5 experiments.

(B) The amount of progranulin secreted by macrophages is macrophage maturation-dependent. Human monocyte-derived macrophages secreted progranulin into the conditioned media and the amount of progranulin protein increased with the differentiation of these cells.

(C) Autocrine regulation of progranulin production. Progranulin could augment the expression of progranulin itself, TNF-alpha (10 ng/mL) and IL-1-beta (10 ng/mL) on macrophages cultured with progranulin. Up-regulation of progranulin expression by progranulin itself, TNF-alpha, or IL-1-beta was observed. Data are the mean ± SE of 5 experiments.

granulin itself, TNF-alpha and IL-1-beta (3.9-, 7.1- and 12.2-fold expressions, respectively).

Progranulin Activates Macrophages and HDL/Apo A-I Suppress as Such Activation

The production of cytokines by macrophages is often regulated in a paracrine or autocrine manner[8]. Next, we examined whether progranulin had effects on macrophages and whether HDL and apo A-I suppress such effects of progranulin. TNF-beta and IL-1-alpha were selected in the present study as representative pro-inflammatory cytokines. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h, and TNF-alpha and IL-1-beta gene expression levels were examined by quantitative PCR using a TaqMan Probe (**Fig. 4A**). Progranulin augmented the expression levels of TNF-alpha and IL-1beta, indicating that progranulin affects macrophages. To confirm the binding of progranulin and HDL/apo A-I, the supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved

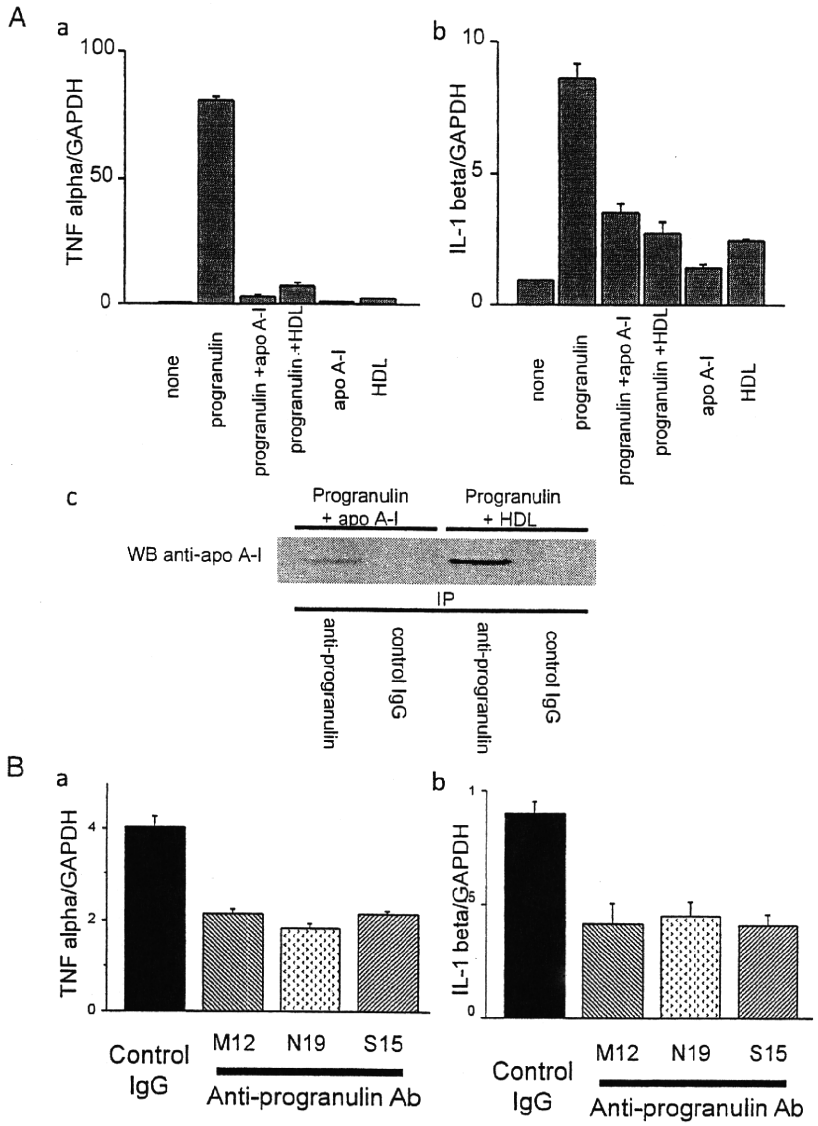

Fig. 4. Effects of progranulin on the expression levels of TNF-alpha and IL-1-beta in macrophages.

(A) Progranulin activated macrophages and HDL/apo A-I suppressed the effects. Seven-day-cultured macrophages were incubated with progranulin-expressing conditioned medium for 24 h and the expression levels of TNF-alpha (a) and IL-1beta (b) were examined with quantitative PCR using TaqMan Probe. Progranulin augmented the expressions of TNF-alpha and IL-1-beta. These properties of progranulin were suppressed by co-incubation with apo A-I (5 μg/mL) or HDL (10 μg/mL). Data are the mean ± SE of 5 experiments. The supernatants of macrophages with progranulin-expressing conditioned medium, and apo A-I or HDL under the same conditions were applied for immunoprecipitation with anti-progranulin. The precipitated samples were resolved by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c).

(B) Anti-progranulin antibodies reduced the expression of TNF-alpha and IL-1beta. Macrophages were cultured in the conditioned medium with anti-progranulin antibody for 24 h. Compared to incubation with control IgG (100 μg/mL), anti-progranulin antibody (clone M12, N9 and S15, 100 μg/mL each) suppressed the expressions of TNF-alpha (a) and IL-1-beta (b). Data are the mean ± SE of 5 experiments.

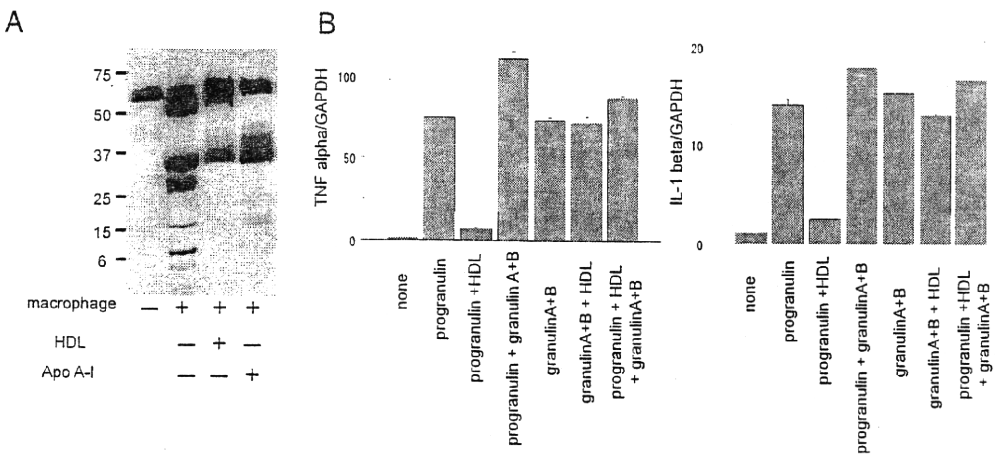

Fig. 5. Progranulin exerts its proinflammatory properties via conversion into granulins.

(A) Progranulin conversion by macrophages. C-terminal-myc-His-tagged progranulin was incubated with or without human macrophages for 24hr. Progranulin was degraded by macrophages and small degraded products were observed, but conversion was suppressed by incubation with HDL or apo A-I.

(B) Suppression of pro-inflammatory properties of progranulin by HDL via inhibition of conversion into granulins. Progranulin could increase the expression of pro-inflammatory cytokines, TNF-alpha and IL-1 beta, and this augmentation was suppressed by incubation with HDL (10 μg/mL). On the other hand, the augmentation effect on the expression of TNF-alpha and Il-1 beta of granulin was not blocked by incubation with HDL. Data are the mean ± SE of 4 experiments.

by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-apo A-I antibody, and then visualized (c). These results indicated that the effects of progranulin were suppressed by co-incubation with HDL or apo A-I via binding.

Anti-Progranulin Antibodies Reduce the Expression of TNF-Alpha and IL-1Beta

To examine whether the effect of progranulin on macrophages is autocrine in nature, fully differentiated macrophages were cultured with anti-progranulin antibody for 24 h. Compared to incubation with control IgG, anti-progranulin antibody (clone M12, N9 and S15) suppressed the expression of TNF-alpha and IL-1-beta (**Fig. 4B**). These results suggest that progranulin is secreted by macrophages and its effect is autocrine in nature, indicating that progranulin is an autoactivating molecule.

Progranulin Exerts its Properties Via Conversion into Proinflammatory Granulins

We examine whether progranulin could be converted into fragments by macrophages and exerted its properties via conversion into proinflammatory granulins. C-terminal-myc-His-tagged progranulin construct was transfected into HEK293T, and the conditioned media were incubated with or without human macrophages for 24 hr. Probond beads were added to the media to capture His-tagged proteins. Next, the incubated probond beads were applied for immunoblotting with anti-myc antibody. C-terminal-myc-His-tagged progranulin was degraded by macrophages and small degraded products were observed but not with HDL incubation (**Fig. 5A**), indicating that prgranulin could be converted by macrophages.

Next, we examined whether granulin could increased the expressions of TNF-alpha and IL-1-beta, and the increment could be suppressed by HDL (**Fig. 5B**). Progranulin could increase the expressions of pro-inflammatory cytokines, TNF-alpha and IL-1-beta, and this augmentation was suppressed by incubation with HDL. On the other hand, the augmentation effect on the expressions of TNF-alpha and Il-1-beta of granulin was not blocked by incubation with HDL (**Fig. 5B**, left and right panels, respectively). These results suggested that progranulin could exert its pro-inflammatory properties via conversion into granulins, and that HDL could suppress the pro-inflammatory properties of progranulin by inhibiting the conversion into granulins.

Discussion

Progranulin, a PC-cell-derived growth factor (PCDGF), or acrogranin, was purified from the conditioned media of transformed cell lines as an auto-

crine growth factor[11]. It is reported to be involved in cancer progression[8], development[12], wound healing[13], and myeloid cell proliferation[14], whereas mutation of progranulin causes frontotemporal dementia[15, 16]. In the present study, we demonstrated that the macrophage-secreted factor was approximately 130 kDa, while the purified protein identified as progranulin was 80 kDa. The 130-kDa HDL binding protein might be a homodimer or heteromer that includes progranulin, which is known to be glycosylated and to have disulfide bonds[11].

Ong and colleagues[14] used myeloid cell lines and reported the overexpression of progranulin in macrophages and monocyte-derived dendritic cells, and that the level of expression was dependent on cell differentiation. Our results also demonstrated that the differentiation of human monocyte-derived macrophages was associated with increased expression levels of progranulin and that progranulin expression was regulated in an autocrine fashion in human monocyte-derived macrophages. We also demonstrated that apo A-I, the major component of HDL, suppressed the conversion of progranulin into pro-inflammatory granulins on human peripheral monocyte-derived macrophages. This is in agreement with others, who indicated that the protein precursor (progranilin) and its processed fragments (granulins) are both bioactive and pro-inflammatory[8].

The role of progranulin in the inflammatory process was initially explored in research on the functions of secretory leukocyte protease inhibitor (SLPI) in wounds[10]. Using a yeast two-hybrid approach, with SLPI as the bait, Zhu and colleagues[10] demonstrated that progranulin is associated with SLPI[10]. This interaction was confirmed by immunoprecipitation experiments demonstrating that progranulin regulates inflammation through a tripartite loop with SLPI, which protects progranulin from proteolysis, and elastase, which digests progranulin between granulin/epithelin domains, generating smaller granulin/epithelin peptides. SLPI blocks this proteolysis, by inhibiting both elastase activity directly and by binding progranulin and sequestering it from the enzyme[10]. Intact progranulin is anti-inflammatory through the inhibition of certain actions of TNF-alpha, while proteolytic peptides may stimulate the production of pro-inflammatory cytokines, such as IL-8[8]. We suppose that HDL/apo A-I have anti-inflammatory effects on macrophages through the formation of a complex with progranulin and prevent the conversion of progranulin into granulins by elastase secreted by macrophages such as SLPI, which is reported as a neutrophil-derived anti-inflammatory factor[10]. In this study, the possibility could be not rejected that progranulin binds HDL particles itself but free apoA-I is dissociated from HDL, which is just a reserve of apoA-I *in vivo* according to the limitations of the experimental conditions. In the near future, our colleagues will demonstrate which of apo A-I, lipid-free apo A-I dissociated from HDL, apo A-I in pre-beta HDL or apo A-I in HDL will bind to progranulin *in vivo*.

An unstable and subsequently ruptured atherosclerotic coronary plaque superimposed on thrombosis constitutes the most common pathological background of acute coronary syndrome[7]. High levels pro-inflammatory cytokines have been found in unstable angina, possibly supporting their role in acute coronary syndrome[7]. Cytokines induce their own expression in an autocrine fashion and also the expression of various adhesion molecules via the cellular transcription factor NF-kappaB[17]. Monocytes adhering to the endothelium and penetrating the plaque (macrophages) are activated by several paracrine/autocrine pro-inflammatory mediators. At this crucial stage, activated macrophages then synthesize and secrete pro-inflammatory cytokines TNF-alpha and IL-1-beta[7]. The human and murine progranulin promoter contains potential inflammation-related promoter elements[18, 19]. Furthermore, TNF-alpha and IL-1-beta activate progranulin gene expression through the NF-kappaB system[18, 19]. The progranulin-granulin loop might play a role in autoactivation of macrophages. Given its actions in atherosclerosis, progranulin may prove a useful clinical target, both for prognosis and therapy.

In summary, we identified progranulin/proepithelin/acrogranin/PCDGF as a macrophage-derived factor whose properties were suppressed by binding to HDL/apo A-I. The normal function of progranulin is complex; the full-length form of the protein has both trophic and anti-inflammatory activities, whereas proteolytic cleavage generates granulin peptide that promotes inflammatory activity. Based on our results, we propose that HDL prevents the conversion of progranulin into its degraded proinflammatory products, granulins; however, the mechanisms involved in HDL-induced suppression of the pro-inflammatory effects of progranulin have not fully elucidated. Further studies are necessary to identify these mechanisms.

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OBSERVATIONS

One-Year Reductions in Body Weight and Blood Pressure, but Not in Visceral Fat Accumulation and Adiponectin, Improve Urinary Albumin-to-Creatinine Ratio in Middle-Aged Japanese Men

Microalbuminuria has been recognized recently as a risk factor for cardiovascular diseases as well as renal failure and is often found in subjects with metabolic syndrome. The relationship between visceral fat accumulation and microalbuminuria, urinary albumin-to-creatinine ratio (UACR), has not been fully clarified. Our cross-sectional study demonstrated that visceral fat accumulation is associated with increases in UACR. However, the accompanying obesity-related risk factors, especially hemoglobin A1c (HbA1c) and elevated blood pressure, strongly increased the risk of UACR (1). We have reported that reductions in both body weight and estimated visceral fat area (eVFA) measured by the bioelectrical impedance analysis method (2) were accompanied by reductions in the number of obesity-related cardiovascular risk factors (3) and increases in serum levels of adiponectin (4) in our longitudinal Amagasaki Visceral Fat Study, in which intensive risk factor–oriented health promotion programs were provided. The present study investigated the relationship between 1-year Δ changes in UACR (ΔUACR) and the estimated visceral fat area (ΔeVFA), and other parameters in these subjects.

The study subjects were 1,539 Japanese males (mean ± SD age 45.8 ± 10.4 years [range 20–68 years]) who were employees of the Amagasaki City Office, had undergone an annual health checkup in both 2006 and 2007, and were not taking any medications for diabetes, hypertension, and dyslipidemia. The study was approved by the human ethics committee of Osaka University, and a signed informed consent form was obtained from each participant. The registration number of the trial at the UMIN is 000002391 (the Amagasaki Visceral Fat Study). Height, weight, and waist circumference (WC) at the umbilical level were measured in standing position. Serum concentrations of adiponectin were measured by the latex particle-enhanced turbidimetric assay. UACR was calculated from a single spot urine specimen. ΔUACR correlated with ΔBMI ($r = 0.077$, $P = 0.0036$) and Δsystolic blood pressure (SBP, $r = 0.063$, $P = 0.0127$), but not ΔHbA1c ($P = 0.0705$), Δtriglyceride ($P = 0.0524$), ΔHDL cholesterol ($P = 0.9906$), ΔLDL cholesterol ($P = 0.5761$), Δestimated glomerular filtration rate (eGFR, Modification of Diet in Renal Disease [MDRD], $P = 0.1724$), ΔeVFA ($P = 0.3026$), and Δadiponectin ($P = 0.9860$). Stepwise multiple regression analysis identified ΔBMI ($F = 6.341$) and ΔSBP ($F = 4.053$) as significant determinants of ΔUACR.

The present study demonstrated that, unlike ΔBMI and ΔSBP, 1-year reduction of eVFA only was not sufficient to improve UACR. This meant a significant reduction in blood pressure, which is downstream of visceral fat accumulation, should be required. We could not find a significant relationship between 1-year changes in UACR and HbA1c, probably because the number of subjects with abnormal HbA1c values (≥5.8%) was small ($n = 130$ of 1,539, 8.4%). Although previous studies reported a relationship between serum levels of adiponectin and albuminuria in obese subjects (5) and general men (1), we could not observe a relationship between 1-year changes in UACR and adiponectin in general men. Monitoring over a longer period of time might be needed. To our knowledge, this is the first report demonstrating the relationship between 1-year changes in UACR and visceral fat accumulation and adiponectin.

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