Discussion

Our study had three major findings in Japanese obese subjects. First, SNP45 in the adiponectin gene was associated with body fat distribution. Second, SNP45 was associated with the development of carotid atherosclerosis. Moreover, SNP45 had an impact on the effect of visceral obesity for the progression of atherosclerosis. Third, there was a gender difference in the effect of SNP45.

First, we demonstrated that the G allele had higher VFA, lower SFA, and a significantly higher V/S ratio compared to the TT genotype in men. Multivariate regression analysis showed that SNP45 was an independent determinant of the V/S ratio. These results indicated that the G allele of SNP45 is a risky genotype of visceral adiposity, resulting in metabolic syndrome. To our knowledge, this is the first study to demonstrate the association of SNP45 with body fat distribution. Some reports have shown that SNP45 contributes to obesity, insulin resistance, or dyslipidemia[10, 18, 19]. In contrast, Ukkola *et al.* reported that SNP45 was found in equal frequency among obese and non-obese Swedish subjects[20]. In French Caucasians, the 45G allele frequency was similar in morbidly obese adults and control subjects[21]. The inconsistency between these reports suggested that SNP45 could not be associated simply with weight or prevalence of obesity, but might contribute to body fat distribution in the process of becoming obese. Since visceral adipose tissue is widely believed to play a key role in the pathogenesis of metabolic abnormalities, the G allele of SNP45 could be an independent risk factor for metabolic syndrome.

Second, another important finding of the present study was the significant association between SNP45 and carotid artery PS in men. A similar trend was observed in women. Multivariate regression analysis showed that SNP45 was an independent determinant of PS. These findings suggest that SNP45 may affect the development of carotid atherosclerosis not only by modulating visceral obesity but also by other pathways.

To the best of our knowledge, PS tends to be associated with the V/S ratio only in the G allele in both men and women. In a previous study, we described a strong association between the V/S ratio and carotid artery PS in Japanese males with metabolic syndrome[22], but patients with the TT genotype were protected from the atherogenic effect of visceral obesity. We hypothesized that visceral obesity might exaggerate the dysregulation of adiponectin properties of the G allele. The mechanism was unclear, but this hypothesis needs confirmation by expression studies.

Third, in this study the degree of the effect of SNP45 on body fat distribution and PS was different between men and women. Adipose tissue is sexually dimorphic in humans, with gender-specific differences in body fat distribution[23, 24]. Gonadal steroids are the major mediator of sex dimorphism of body composition in adults[25, 26]. Estrogen regulates both the metabolism and location of adipose tissue and plays a role in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation[27]. Furthermore, in recent studies, Clegg *et al.* reported that gonadal steroids mediate body fat distribution and interact with the integrated adiposity messages conveyed to the brain[28]. Taken together with previous studies, our findings suggest that estrogen may interact with the adiponectin gene in adipocyte and modulate the effect of SNP45.

In addition, estrogen is known to have a cardioprotective effect. *In vivo* evidence suggests that the effect of estrogen on adhesion molecules is mediated by the inhibition of nuclear factor (NF)-κB DNA binding[29, 30]. As adiponectin has been shown to suppress the expression of class A scavenger receptors in macrophages, to affect the NF-κB pathway and to inhibit monocyte adhesion to aortic endothelial cells[6-8], atherogenic properties of the G allele may be suppressed by the effect of estrogen. Estrogen could interact with SNP45 and modulate the atherogenic function of adiponectin, but further large studies are needed to confirm the mechanism of gender-specific differences in the effect of SNP45.

The mechanistic relationship between SNP45 and both body fat distribution and the progression of atherosclerosis is unclear. SNP45 is located in exon 2 of the adiponectin gene and does not cause an amino acid change (GGT to GGG, Gly15Gly). One possibility is that SNP45 may have linkage disequilibrium with other undiscovered SNPs of the adiponectin gene having an effect on adiponectin expression, secretion, structure, or action. Another possibility is that SNP45 located in exon 2 is relatively close to the exon-intron boundary which may affect splicing machinery and effect adiponectin expression. The G allele of SNP45 may act through decreased adiponectin expression, which may cause increased visceral adipose tissue. Indeed, in Japanese type 2 diabetes, SNP45 is reported to be associated with reduced adiponectin levels[11]. Similar findings have been shown in an other study[31]. Furthermore, recent studies have reported various adiponectin functions as an adipocyte differentiation factor, helping to maintain equilibrium adipocyte size, as an autocrine/paracrine factor in adipose tissue and as a participating factor in the regulation of adipocyte metabolism and adipose tissue mass. In 3T3-L1 preadipocytes,

adiponectin overexpression accelerates cell proliferation and differentiation, while in mature adipocytes, autocrine adiponectin increases glucose uptake and lipid accumulation[32]. Transgenic overexpression of adiponectin in the physiological range induced morbid obesity without insulin resistance in ob/ob mice[21]. These reports indicated that hyperadiponectinemia may induce simple obesity with more subcutaneous fat accumulation, while decreased adiponectin levels may induce visceral obesity. Interestingly, the present study showed that hypoadiponectinemia was the third independent determinant of the V/S ratio. Due to these previous findings combined with our present study, the G allele might be genetically determined to have hypoadiponectinemia, contributing to the progression of visceral obesity. In contrast, the TT genotype might favor the accumulation of subcutaneous adipose tissue through hyperadiponectinemia, preventing insulin resistance, and eventually metabolic syndrome.

Adiponectin exists largely as low molecular weight (LMW) hexamers and high molecular weight (HMW) multimers[32, 33]. Recent article showed that the ratio of HMW to total adiponectin was responsible for metabolic effects[34]. Another study showed that HMW adiponectin was an important factor in metabolic syndrome[35]. Therefore, the alternative possibility of the atherogenic effect of SNP45 is that the proportion of HMW adiponectin might decrease in the G allele of SNP45, leading to atherosclerosis. As we measured total adiponectin and did not assess multimeric forms of adiponectin, further study is needed.

In conclusion, we demonstrated that SNP45 was associated with body fat distribution and PS of carotid arteries. The TT genotype is a protective genotype from metabolic syndrome and atherosclerosis progression in Japanese obese subjects. The mechanism by which SNP45 affects body fat distribution and the development of atherosclerosis has not been clarified at present. Further investigations will be needed to elucidate the functional mechanism of this polymorphism.

Acknowledgements

This study was partially supported by the Second Department of Internal Medicine, Kanazawa University Hospital. We thank Mrs. Reiko Ikeda for her excellent technical assistance.

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High Frequency of a Retinoid X Receptor γ Gene Variant in Familial Combined Hyperlipidemia That Associates With Atherogenic Dyslipidemia

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Objective—The genetic background of familial combined hyperlipidemia (FCHL) has not been fully clarified. Because several nuclear receptors play pivotal roles in lipid metabolism, we tested the hypothesis that genetic variants of nuclear receptors contribute to FCHL.

Methods and Results—We screened all the coding regions of the PPARα, PPARγ2, PPARδ, FXR, LXRα, and RXRγ genes in 180 hyperlipidemic patients including 60 FCHL probands. Clinical characteristics of the identified variants were evaluated in other 175 patients suspected of coronary disease. We identified PPARα Asp140Asn and Gly395Glu, PPARγ2 Pro12Ala, RXRγ Gly14Ser, and FXR −1g−>t variants. Only RXRγ Ser14 was more frequent in FCHL (15%, $P<0.05$) than in other primary hyperlipidemia (4%) and in controls (5%). Among patients suspected of coronary disease, we identified 9 RXRγ Ser14 carriers, who showed increased triglycerides (1.62±0.82 versus 1.91±0.42 [mean±SD] mmol/L, $P<0.05$), decreased HDL-cholesterol (1.32±0.41 versus 1.04±0.26, $P<0.05$), and decreased post-heparin plasma lipoprotein lipase protein levels (222±85 versus 149±38 ng/mL, $P<0.01$). In vitro, RXRγ Ser14 showed significantly stronger repression of the lipoprotein lipase promoter than RXRγ Gly14.

Conclusion—These findings suggest that RXRγ contributes to the genetic background of FCHL. (***Arterioscler Thromb Vasc Biol.* 2007;27:923-928.**)

Key Words: apolipoproteins ■ gene mutations ■ lipoprotein lipase ■ familial combined hyperlipidemia ■ nuclear receptors

Familial combined hyperlipidemia (FCHL) is the most common form of inherited hyperlipidemia. FCHL shows strong genetic susceptibility resembling an autosomal dominant disease,[1–3] but most of the underlying causal mechanisms remain to be elucidated. Lipoprotein lipase (LPL) has been implicated as one of the genes that modify the lipid phenotype in FCHL.[4,5] "Intra-individual variability" of the lipoprotein phenotype is often included as a criterion in diagnosis.[6] However, a recent prospective study of FCHL families suggests that this variability may even include normolipidemic periods in affected subjects.[7] This feature indicates that FCHL could be a "disease of regulation" rather than a genetic defect in certain peripheral components of lipid metabolism.

Nuclear receptors are transcription factors that can be activated by specific ligands. Recent studies have shown that nuclear receptors, especially retinoid X receptor (RXR) and its heterodimerization partners,[8] play important roles in maintenance of lipid homeostasis on their activation by a variety of ligands derived from dietary cholesterol and fatty acids.[9] The peroxisome proliferator-activated receptors (PPARs) family, the oxysterol sensor liver X receptor (LXR), and the bile acid sensor farnesoid X receptor (FXR) are all involved in control of plasma lipid concentrations.[10] Thus, we tested the hypothesis that variants of these nuclear receptors, ie, PPARα, PPARγ2, PPARδ, LXRα, FXR, and RXRγ, could constitute part of the genetic background of atherogenic dyslipidemia, particularly of FCHL.

Methods

Subjects

The study design consists of 2 parts. First, we screened for frequent variants in the nuclear receptor candidate genes among 180 patients with primary hyperlipidemia, including 60 unrelated patients with FCHL (clinical characteristics are presented in supplemental Table I, available online at http://atvb.ahajounals.org). Patients with familial

Original received August 28 2006; final version accepted January 6, 2007.

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000258945.76141.8a**

hypercholesterolemia and secondary hyperlipidemia were excluded. Diagnosis of FCHL was based on the fulfillment of all of the following three criteria: (1) Phenotype IIb, IIa, or IV hyperlipidemia according to the Fredrickson classification; (2) Presence of phenotype IIb, IIa, or IV hyperlipidemia in a first-degree relative and at least one family member with phenotype IIb; (3) Exclusion of familial hypercholesterolemia. Two hundred ninety-eight anonymous samples from healthy males were used as controls for frequency analysis of identified mutations. All blood samples in this study were obtained after an overnight fast.

Second, we evaluated the clinical impact of potentially relevant variants in another 175 patients who were suspected of having coronary artery disease based on any of the following reasons: ECG abnormalities, cumulative coronary risk factors, and/or chest symptoms. The group included 105 patients who had undergone coronary angiography. Patients with familial hypercholesterolemia were excluded because of their clear genetic background for hyperlipidemia. The extent and severity of atherosclerotic changes in coronary angiography were assessed by assigning scores to each of the 15 segments, according to the classification of the American Heart Association Grading Committee. The coronary stenosis index (CSI) was defined as the sum of the following scores[11]: A normal coronary angiogram was graded 0, stenosis of less than 25% was graded 1, 25% to 50% stenosis was graded 2, 50% to 75% stenosis was graded 3, and more than 75% stenosis was graded 4. CSI is a useful index for evaluating mild-moderate coronary atherosclerotic changes.

All the subjects and controls enrolled were inhabitants of the Hokuriku district of Japan. Written informed consent was obtained from each of the subjects. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University.

Laboratory Analyses

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, apolipoproteins, glucose, and thyroid hormones were measured according to standard clinical laboratory techniques. HDL-cholesterol fractions were obtained by dextran sulfate-magnesium chloride precipitation and assayed using a commercial kit (Daiichi, Tokyo, Japan).[12] Separation of lipoproteins by ultracentrifugation was performed as described by Havel et al.[13] Plasma remnant-like particle (RLP)-cholesterol was determined by immunoabsorption using the commercial RLP-C JIMRO kit.[14] Plasma cholesteryl ester transfer protein (CETP) concentrations were determined by enzyme-linked immunosorbent assay using the monoclonal antibody TP2 and a rabbit polyclonal antibody raised against recombinant human CETP.[15] For LPL assessment, blood samples were obtained 10 minutes after an intravenous injection of 30 IU heparin/kg body weight. LPL activity was measured using radiolabeled triolein emulsion after hepatic lipase (HL) inhibition by SDS as previously described.[16] LPL mass was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibody against LPL (Daiichi Pure Chemicals Co Ltd, Tokyo, Japan).[17]

Genetic Analyses of Candidate Genes

Genomic DNA was isolated from peripheral white blood cells using standard phenol-chloroform extraction techniques. We screened all the coding regions of PPARα (NM_032644), PPARδ (NM_006238), PPARγ2 (NM_015869), LXRα (NM_005693), FXR (NM_005123), and RXRγ (NM_006917) genes with flanking exon-intron boundaries by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) using the DCode system (Bio-Rad), which is highly accurate in detecting changes in nucleic acids.[18] The structural organization and nucleotide sequences of these genes were retrieved from the gene database of NCBI. Lists of all GC-clamped primers used in DGGE analysis are available online (supplemental Table II). Samples with a variant detected by DGGE analysis were directly sequenced on an ABI310 analyzer (Applied Biosystems). PCR-restriction-fragment-length polymorphisms analysis on the RXRγ Ser14 variant was performed with the primers 5′-AGCCGAGAGAGGCGGTAATA-3′ (forward) and 5′-TACAGGTCCACGCAGTGAAG-3′ (reverse) in patients suspected of coronary artery disease. Digestion with *Alu*I resulted in a 76-bp fragment for Ser allele and a 120-bp fragment for Gly allele.

Cell Culture and Transfection Assays

Cos7 cells were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, glutamine, and nonessential amino acids (Gibco BRL, Invitrogen). The medium was changed every 48 hours. Cos7 cells were transfected using FuGENE 6 reagent (Roche): 150 ng of the indicated LPL firefly luciferase reporter plasmid (a generous gift of Dr B. Staels, Institut Pasteur de Lille, France), that contains the proximal 466-bp of the human LPL promoter in front of the ATG cloned into the *Hind*III site of the pGL3 plasmid, was cotransfected with or without 100 ng of the human RXRγ expressing vector (a generous gift of Dr W. Lamph, Ligand Pharmaceuticals Inc, San Diego, Calif). After an overnight incubation, cells were incubated with medium containing 10% FCS with or without the retinoid LGD1069, (1 μmol/L, Sigma) and luciferase activity was assayed 48 hours later using an Orion luminometer (Berthold). Transfection studies were performed at least 3 times in triplicate. Transfection efficiency was monitored by cotransfection of 150 ng of a SV40-driven β-galactosidase expression plasmid. A positive RXRE TKpGL3 construct was made by cloning 3 copies of the direct repeat AGGTCA spaced by 5 nucleotides in the TKpGL3 plasmid.

Plasmid Site-Directed Mutagenesis

Nucleotide substitution was introduced in the plasmid expressing human RXRγ using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) and the primer 5′-CATGAAGTTTCCCGCAAGCTATGGAGGCTCCCCTGG C-3′ in which the nucleotide in bold indicates the mutation.

Statistical Analysis

The frequency distribution of genotypes was compared using standard χ^2 tests. Student t test was used for normally distributed parameters and the Kruskal–Wallis test was used for non-normally distributed parameters: triglycerides levels, LPL levels, and CSI. JMP 5.1.2 software (SAS Institute Inc) was used for statistical calculation.

Results

Identified Variants in Nuclear Receptor Genes

With PCR-DGGE analysis, we identified 4 variants with amino acid changes, ie, Asp140Asn and Gly395Glu in the PPARα gene, Pro12Ala in the PPARγ2 gene, Gly14Ser in the

TABLE 1. Frequencies of Nuclear Receptor Genes Variants Identified in This Study

	FCHL n=60	Other Hyperlipidemia n=120	General Population n=298	*P* Value
PPARαGly395Glu				
Glu395	3 (5%)	1 (0.8%)	6 (2%)	ns
PPARαAsp140Asn				
Asn140	2 (3%)	1 (0.8%)	2 (0.6%)	ns
PPARγ2Pro12Ala				
Ala12	5 (8%)	10 (8%)	20 (7%)	ns
FXR −1g−>t				
−1g/t	19 (32%)	34 (28%)	108 (36%)	ns
−1t/t	2 (3%)	6 (5%)	27 (9%)	ns
RXRγGly14Ser				
Ser14	9 (15%)	5 (4%)	15 (5%)	0.03

TABLE 2. Clinical Characteristics of Patients With RXRγ Variant

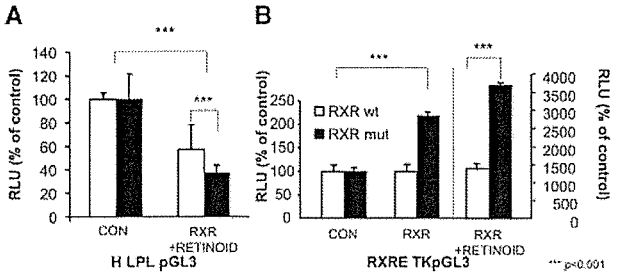
	RXRγ Gly14Ser		
	Gly/Gly	Gly/Ser	*P* Value
Number (M/F)	166 (78/88)	9 (5/4)	
Age, y	58±15	58±7	ns
BMI, kg/m^2	23.4±5	23.9±2	ns
Smoking, %	36	33	ns
Total cholesterol, mmol/L	5.98±1.4	5.96±1.55	ns
Triglycerides, mmol/L	1.62±0.82	1.91±0.42	*P*<0.05
HDL cholesterol, mmol/L	1.32±0.41	1.04±0.26	*P*<0.05
LDL cholesterol, mmol/L	3.94±1.27	4.07±1.45	ns
HDL2 cholesterol, mmol/L	0.78±0.28	0.54±0.10	*P*<0.05
HDL3 cholesterol, mmol/L	0.44±0.10	0.39±0.08	ns
ApoA-I, g/L	1.38±0.31	1.18±0.18	ns
ApoA-II, g/L	0.32±0.06	0.28±0.05	*P*<0.05
ApoB, g/L	1.31±0.38	1.35±0.31	ns
ApoC-II, g/L	0.06±0.02	0.05±0.02	ns
ApoC-III, g/L	0.11±0.05	0.10±0.03	ns
ApoE, g/L	0.06±0.02	0.05±0.01	ns
RLP cholesterol, mmol/L	0.15±0.10	0.21±0.10	*P*<0.01
CETP, mg/L	2.52±0.82	2.48±0.73	ns
Intraindividual lipoprotein phenotype variability, %	27	88	*P*<0.01
Fasting glucose, mmol/L	5.72±1.39	5.33±0.72	ns
HbA1c, %	5.6±1.0	5.8±1.0	ns
Fasting insulin, pmol/L	70.8±90.3	52.1±1.0	ns
HOMA-IR	2.28±2.1	2.19±1.7	ns
Diabetes, %	28	33	ns
HL activity, U/L	0.24±0.09	0.26±0.07	ns
LPL activiy, U/L	0.11±0.06	0.08±0.03	*P*<0.05
LPL mass, ng/mL	222±85	149±38	*P*<0.01
FT3, pmol/L	0.42±0.01	0.044±0.01	ns
FT4, pmol/L	15.2±5.15	13.3±2.57	ns
TSH, μU/mL	2.31±2.8	2.53±0.9	ns
Number (M/F)	100 (50/50)	5 (4/1)	
CSI	12.3±10	21.4±6	*P*<0.05
			mean±SD

BMI indicates body mass index; HOMA-IR, homeostasis model assessment; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone.

RXRγ gene, and 1 nucleotide substitution in a flanking coding region, ie, FXR −1g−>t variant. The PPARγ2 Pro12Ala polymorphism has already been well-described,[19] whereas the others represent novel variants identified in this study. In humans, variants in the RXRγ gene have been associated with elevated triglyceride levels in familial type 2 diabetes, but none of these variants showed an altered coding sequence.[20] Therefore, this is the first description of a RXRγ variant with an amino acid substitution. In the PPARα gene, the Leu162Val variant has been reported in Western countries,[21] but this variant was not identified in this study. We also identified some silent nucleotide substitutions, ie, 891C−>G (rs13306747) and 1431C−>T (rs1724155) in the PPARγ2 gene, 1233C−>T (rs9658166) in the PPARδ gene, and 1134A−>G (rs1131379) in the LXRα gene. We did not identify variants with amino acid changes in the PPARδ and LXRα genes. We further investigated the variants with amino acid substitutions and the −1g−>t FXR variant, because of the likelihood that these induced altered physiological function.

Higher Frequency of RXRγ Variant in FCHL

We evaluated the frequencies of the 5 identified polymorphisms in subjects with FCHL, subjects with other forms of primary hyperlipidemia and in the general population (Table 1). Only the RXRγ Ser14 variant was found to be significantly more frequent in FCHL patients (15%) compared with that in other forms of primary hyperlipidemia (4%) or the general population (5%).



A, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of the LPL promoter. B, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of a positive RXRE cloned in the TKpGL3 plasmid.

Atherogenic Plasma Lipids Profiles and Coronary Atherosclerosis Associated With the RXRγ Ser14 Variant

To establish the impact of the identified RXRγ variant on metabolic parameters and on coronary atherosclerosis, we evaluated anthropometric parameters and laboratory data from 175 patients suspected of coronary disease. The RXRγ Ser14 variant was identified in 9 patients, all of whom were heterozygotes. Eight of the RXRγ Ser14 carriers had hyperlipidemia, while the remaining 1 demonstrated an isolated low HDL cholesterol level. Clinical characteristics of patients with or without the RXRγ Ser14 allele are shown in Table 2. There was no difference in age or body mass index between the two groups. In their lipid profiles, RXRγ Ser14 carriers had higher TG, lower HDL cholesterol especially in the HDL2 subfraction, and lower apolipoprotein A-II levels. There was no difference in CETP protein levels between the groups. Furthermore, we found that the RLP cholesterol level was significantly higher in the RXRγ Ser14 carriers than in the wild-type. Subjects with this variant also showed significantly lower LPL activities and protein levels in post-heparin plasma. Separation of lipoproteins demonstrated that the Ser14 carriers had higher TG levels in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions, higher cholesterol levels in VLDL, and lower cholesterol levels in HDL (supplemental Table III).

Two RXRγ Ser14 carriers were diagnosed as FCHL (22%), and 2 additional carriers were suspected of FCHL with hyperlipidemic siblings without information on first-degree relatives. Among non-carriers, 22 of 166 patients were diagnosed as FCHL (13%). One hundred twenty-five patients suspected of coronary disease showed hyperlipidemia and the intraindividual variability of lipoprotein phenotype was significantly more frequent in RXRγ Ser14 carriers (7 of 8 hyperlipidemic patients; 88%) than in wild-type (32 of 117 hyperlipidemic patients; 27%, Table 2).

There was no significant difference in the thyroid hormone levels between the two groups.

Four males and 1 female were identified as RXRγ variant carriers among 105 patients who underwent coronary angiography. The carriers of RXRγ Ser14 demonstrated significantly higher CSI than those with the wild-type (Table 2).

RXRγ Variant Represses More Efficiently the LPL Promoter Activity

Because RXRγ Ser14 carriers showed significantly lower LPL activities and protein levels in post-heparin plasma, we hypothesized that activated-RXRγ downregulates LPL gene expression by a transcriptional mechanism and that RXRγ variant is more effective in repressing the LPL promoter activity. Therefore, transfection assays were performed using the LPL promoter cotransfected with either wild-type RXRγ or the variant (Figure). Interestingly, RXRγ Gly14 significantly repressed (−40%) the LPL promoter activity, whereas the RXRγ Ser14 repressed even more strongly (−60%, $P<0.001$, Figure A). Moreover, the RXRγSer14 was a more potent activator of a positive RXRE cloned in front of a TKpGL3 plasmid (note the different scales in Figure B). Taken together, our results indicate that RXRγ downregulates human LPL gene expression, at least partially by a transcriptional mechanism, and that the newly identified RXRγ variant is a more potent repressor than the wild-type in this respect, as well as a more potent transactivator of a positive RXR response element.

Gain of Function Variant of PPARα and Increased LDL-C Levels

The carriers of the PPARα variant Gly395Glu tended to have higher frequency in the FCHL population, although not statistically significant. Four subjects were identified as PPARα Glu395 carriers in the coronary artery disease-suspected group and showed significantly higher LDL-cholesterol levels (supplemental Table IV). On in vitro functional analysis, Glu395 showed a moderately but significantly increased transcriptional activity compared with wild-type PPARα (supplemental Figure I, available online at http://atvb.ahajournals.org). The previously described Leu162Val variant of the PPARα gene has been shown to give gain of function in in vitro,[24] has been associated with raised LDL-cholesterol levels.[21,22] Our results appear to be in accordance with these previous reports.

Discussion

The main findings of the present study are the following: (1) identification of novel polymorphisms in plasma lipid levels-associated nuclear receptor genes, (2) a higher frequency of the RXRγ gene variant Gly14Ser in subjects with FCHL, (3) RXRγ Ser14 variant carriers showed more atherogenic dyslipidemia associated with coronary atherosclerosis, (4) the RXRγ variant showed a stronger response to its ligand in repression of the LPL promoter than the wild-type RXRγ.

RXRs are major heterodimerization partners of nuclear receptors such as PPARs, LXRs, and FXR. Three RXR isotypes have been identified: RXRα, RXRβ, and RXRγ. Synthetic RXR ligands induce hypertriglyceridemia through decreased clearance of VLDL by LPL-dependent pathways,[23,24] except in 1 study.[25] In contrast to the embryonic lethality observed in RXRα- and RXRβ-deficient mice, RXRγ-deficient mice develop apparently normal.[26] Yet, RXRγ-deficient mice showed reduced fasting plasma TG levels and increased skeletal muscle LPL activity when fed a high fat diet.[27] The human RXRγ gene is located on chro-

mosome 1q21-q23, ie, the so-called "FCHL locus",[28] and both linkage analysis and a twin study have indicated that the RXRγ gene is linked with dyslipidemia in Chinese and German families.[29,30]

To our knowledge, there are only few data concerning the physiological roles and targets of RXRγ in humans. The RXRγ gene is mainly expressed in skeletal muscles, central nervous system, skin, intestine, and lung. In the present study, LPL protein mass and activity were significantly decreased in RXRγ variant carriers. Because LPL is mainly expressed in adipose tissues and in skeletal muscles, we assume that this is attributable to the fact that the presence of the RXRγ variant affects LPL expression in skeletal muscles. RXRγ mRNA is detectable in adipose tissue only at a low level,[31] but it has been reported that RXRγ could replace RXRα in heterodimerization with PPARγ in adipose tissue.[32] Therefore, there is a possibility that RXRγ variant expression in adipose tissue contributes to the changes in LPL.

It has been reported that RXRγ-deficient mice show a 17% increase in serum thyroid hormone (T4) and a 20% increase in thyroid-stimulating hormone (TSH) levels.[33] In the present study, thyroid hormone levels did not appear to differ sufficiently between variant carriers and non-carriers to explain the differences observed in lipid levels.

It has been shown that low LPL levels contribute to disorders associated with TG-rich lipoprotein catabolism with low HDL, especially in HDL2,[34,35] and are associated with increased risk for future coronary disease.[36] Thus, the low LPL could well contribute to the increase in TG and the decrease in HDL-cholesterol levels in subjects with the RXRγ variant.

We assessed the functional consequence of the RXRγ Ser14 variant in vitro. The activation function-1 (AF-1) domain of RXRγ is located between amino acids 1 and 103, and is required for optimal ligand-dependent transactivation of RXR response element.[37] Fourteen amino acids are located within the AF-1 domain and are conserved among humans, mice, and chickens. In a transfection assay, RXRγ Ser14 repressed LPL promoter activity more strongly than the wild-type RXRγ. In addition, the Ser14 variant was a more potent inducer of a positive RXR response element. Therefore, we speculate that the Ser14 variant induces a better recruitment and/or stabilization of RXR cofactors. Further studies will be required to understand the precise molecular mechanism(s) involved in the LPL regulation by RXRγ Ser14.

Within the so-called FCHL locus, on chromosome 1q21-q23, several genes have been reported to be associated with the FCHL phenotype[28,30,38] and with type 2 diabetes.[39] First, the thioredoxin interacting protein gene was shown to be associated with combined hyperlipidemia in mice, but no disease-causing mutation has been found in humans so far.[40,41] Currently upstream stimulatory factor 1 (USF1) is considered the most promising candidate gene of FCHL.[42] In the USF1 gene, no amino acid substitution has been identified in the coding regions, but single nucleotide polymorphisms in the 3′untranslated region and in intron 7 have been reported to be associated with FCHL, metabolic syndrome, or type 2 diabetes mellitus quite reproducibly.[43–45] However, populations did not show any such association have also been reported.[46–48] These reports emphasize the complexity of phenotypic expression in multi-factorial diseases such as FCHL. RXRγ had been reported to show an association with TG and cholesterol levels on linkage analysis,[29,30] and we identified novel RXRγ variant that associated with atherogenic dyslipidemia. However, the changes in lipid levels attributable to the RXRγ variant alone were not sufficient to cause FCHL. Thus, we suggest the RXRγ gene variant to be a strong modifier rather than a causative gene in development of the FCHL phenotype.

In conclusion, the present study suggests that a variant of RXRγ gene contributes to genetic dyslipidemia, including FCHL, based on the increased frequency of this variant in FCHL, its association with an atherogenic lipid profile, and initial functional studies.

Acknowledgments

The authors thank Sachio Yamamoto for technical assistance. Drs William W. Lamph and Bart Staels are kindly acknowledged for the generous gift of plasmids.

Sources of Funding

This work has been supported by a scientific research grant from the Ministry of Education, Science, and Culture of Japan (No.17790603) and ONO Medical Research Foundation. Thierry Claudel was supported by Grant 2002B017 from the Netherlands Heart Foundation.

Disclosures

None.

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Clinical Science (2006) 111, 325–331 (Printed in Great Britain) doi:10.1042/CS20060088

CETP (cholesteryl ester transfer protein) promoter − 1337 C > T polymorphism protects against coronary atherosclerosis in Japanese patients with heterozygous familial hypercholesterolaemia

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ABSTRACT

CETP (cholesteryl ester transfer protein) and HL (hepatic lipase) play a role in the metabolism of plasma lipoproteins, but the effects of *CETP* and *LIPC* (gene encoding HL) genotypes on coronary atherosclerosis may be dependent on LDL (low-density lipoprotein)-receptor activity. Recently, the − 1337 C > T polymorphism in the *CETP* gene has been reported in REGRESS (Regression Growth Evaluation Statin Study) to be a major determinant of promoter activity and plasma CETP concentration. In the present study, we have investigated the effects of the *CETP* promoter − 1337 C > T and *LIPC* promoter − 514 C > T polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in 206 patients (154 males) with heterozygous FH (familial hypercholesterolaemia). To evaluate coronary atherosclerosis, we used CSI (coronary stenosis index) calculated from coronary angiograms. The *CETP* − 1337 T allele was less frequent in subjects with a CSI ⩾ 14 (mean value) in the group with coronary artery disease ($P = 0.04$, as determined by χ^2 test). ANOVA revealed that HDL-C (high-density lipoprotein-cholesterol) and triacylglycerol (triglyceride) levels were not significantly higher in the presence of the *CETP* promoter − 1337 T allele. Combined with *LIPC* promoter polymorphisms, HDL-C levels were highest and CSI were lowest with *CETP* − 1337 CT + TT and *LIPC* − 514 CC genotypes, but a significant interaction was not shown. A multiple logistic regression analysis revealed that, in patients with coronary atherosclerosis, the *CETP* − 1337 CC genotype was a significant genetic risk factor in FH (odds ratio = 2.022; $P = 0.0256$). These results indicate that the *CETP* promoter − 1337C > T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels.

Key words: cholesteryl ester transfer protein (CETP), coronary artery disease, familial hypercholesterolaemia, hepatic lipase, single nucleotide polymorphism.

Abbreviations: AP, angina pectoris; Apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; CSI, coronary stenosis index; FH, familial hypercholesterolaemia; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; MI, myocardial infarction; NCBI, National Center for Biotechnology Information; REGRESS, Regression Growth Evaluation Statin Study.

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INTRODUCTION

CETP (cholesteryl ester transfer protein) is a key player in the metabolism of major plasma lipoproteins. CETP mediates the transfer of cholesteryl esters from HDL (high-density lipoprotein) to Apo (apolipoprotein) B-containing lipoproteins in exchange for triacylglycerols (triglycerides) [1]. CETP activities are known to be highly affected by genetic factors. For example, individuals with homozygous CETP deficiency have high HDL-C (HDL-cholesterol) levels and low LDL-C [LDL (low-density lipoprotein)-cholesterol] levels, and have no evidence of premature atherosclerosis [2]. Also, *CETP* gene polymorphisms, especially the *Taq*IB polymorphism identified in intron 1, is reported to be highly associated with plasma CETP concentrations and HDL-C levels. Moreover, recent meta-analyses revealed that this polymorphism is associated with the incidence of CAD (coronary artery disease) [3–7]. However, this polymorphism is unlikely to be functional by itself, instead representing a surrogate marker of functional variants of the *CETP* gene [8]. Indeed, previous studies have shown that the *CETP* promoter −629 A > C polymorphism has almost complete linkage disequilibrium with the *Taq*IB polymorphism [9,10], and that this polymorphism is associated with CAD [11]. On the other hand, we have reported [8] that the haplotype block consisting of −2668 G/A, −2505 C/A, −1337 C/T and the shortest (gaaa) repeat had a stronger association than *Taq*IB2 or −629 A/C with low plasma CETP concentrations and high HDL-C levels in healthy Japanese males. Moreover, functional interaction between −629 C/A, −971 G/A and −1337 C/T polymorphisms in the *CETP* gene is a major determinant of promoter activity and plasma CETP concentration in REGRESS (Regression Growth Evaluation Statin Study) [12].

In addition to CETP, HL (hepatic lipase) also plays a crucial role in the metabolism of plasma lipoproteins. HL is involved in the hydrolysis of triacylglycerol and phospholipids in IDL (intermediate-density lipoprotein) and large LDL particles to form smaller and denser LDL particles, and also plays a major role in promoting the conversion from HDL_2 into HDL_3 particles [13]. The effects of the *LIPC* genotype (the gene encoding HL) on atherosclerosis have been controversial [14], and may be dependent on LDL-receptor activity.

FH (familial hypercholesterolaemia) is an autosomal-dominant disorder characterized by primary hypercholesterolaemia with tendon xanthomas and premature CAD caused by mutations in the LDL receptor [15,16]. Mortality from CAD is reported to be several times higher in subjects with heterozygous FH than in the general population [15,16]. There are several reports that polymorphisms or mutations in the *CETP* gene influence the clinical characteristics of FH subjects [17,18]. Carmena-Ramon et al. [17] reported that in FH the *Taq*IB2 allele was associated with higher HDL-C and ApoAI levels. On the other hand, our previous study [18] showed that increased HDL-C levels caused by a heterozygous CETP deficiency was insufficient to prevent CAD in FH.

With this background, the present study investigated the effects of *CETP* promoter −1337 C > T and *LIPC* promoter −514 C > T polymorphisms on coronary atherosclerosis in Japanese patients with heterozygous FH.

METHODS

Study participants

We enrolled 206 consecutive Japanese patients with heterozygous FH (26–83 years old; 154 males) who attended our hospital. FH was diagnosed when one of the following two criteria was met: (i) primary hypercholesterolaemia [> 5.96 mmol/l (> 230 mg/dl) in any age group] in a patient with tendon xanthomas, or (ii) primary hypercholesterolaemia with a definitive diagnosis of FH in any first-degree relative [19]. All the females were postmenopausal, as defined by the absence of menstruation for > 6 months or having attained an age of ⩾ 60 years. Those with surgical menopause were excluded. For patients with MI (myocardial infarction), the age at the first event was recorded, whereas for patients with AP (angina pectoris), the age at which coronary angiography was performed was recorded. Inclusion criteria for this study were FH patients who were examined by coronary angiography because of chest symptoms and/or a positive exercise test before lipid-lowering therapy was initiated. Individuals who had thyroid disease, levels of triacylglycerol ⩾ 4.52 mmol/l (> 400 mg/dl) or who received lipid-lowering agents, corticosteroid or oestrogen hormone replacement therapy were excluded. All patients provided informed consent for participation in the present study, which was approved by the Ethical committee of Kanazawa University Graduate School of Medical Science.

Assessment of CAD

For the evaluation of CAD, we used CSI (coronary stenosis index) to quantify the severity of coronary atherosclerosis. The severity of stenotic changes was assessed by a score assigned to each of the 15 segments according to the classification of the American Heart Association Grading Committee. A normal coronary angiogram was graded as 0, stenosis of < 25 % was graded as 1, 25–50 % stenosis was graded as 2, 50–75 % stenosis was graded as 3, and > 75 % stenosis was graded as 4. CSI was defined as the sum of these scores in all 15 segments, producing a maximal value of 60 [15]. In the present study, MI was diagnosed in 56 subjects with

heterozygous FH (48 male), and AP was diagnosed in 53 subjects with heterozygous FH (all male). The mean CSI was 14.0 ± 11. The mean CSI in subjects who were diagnosed with MI and AP was 20, whereas the mean CSI in those subjects who were without clinical symptoms of CAD was 8. In our previous study [15], we observed that the age of coronary artery stenosis detectable by angiogram occurs after 17–25 years of age in male and female subjects with heterozygous FH. In the present study, 86 % of the subjects with MI and AP had a CSI > 14, whereas 80 % of subjects without clinical symptoms of CAD had a CSI < 14. Therefore we diagnosed CAD as being present when CSI was > 14.

Assessment of conventional risk factors

Data for BMI (body mass index), smoking history, alcohol drinking, blood pressure, diabetes status and lipid profile were collected. Hypertension was considered to be present if any antihypertensive treatment had been instituted, if systolic blood pressure was > 160 mmHg or diastolic blood pressure > 95 mmHg. Diabetes mellitus was diagnosed if fasting plasma glucose was ⩾ 6.70 mmol/l (> 120 mg/dl) or ⩾ 11.10 mmol/l (> 200 mg/dl) at 120 min after 75 g of oral glucose loading, or if HbA_{1c} (glycated haemoglobin) was ⩾ 6.5 %. For smoking status, we defined subjects who smoked ⩽ 10 cigarettes/day as non-smokers, past smokers as ex-smokers and current smokers.

Laboratory analysis

Blood samples were collected from subjects after 12 h of fasting before starting lipid-lowering agents. Total cholesterol, triacylglycerols and HDL-C levels were determined by standard enzymatic methods. LDL-C levels were calculated using the Friedewald formula [20]. Plasma CETP levels were determined by sandwich ELISA, as described previously [21].

Determination of *CETP* and *LIPC* promoter polymorphisms

Genomic DNA was isolated and purified from peripheral white blood cells. The *CETP* promoter − 1337 C > T polymorphism and the *LIPC* promoter − 514 C > T polymorphism (− 480 in older reports) were analysed by PCR-RFLP (restriction-fragment-length polymorphism) methods, as described previously [8,22]. Accession numbers are as follows: *CETP*, gene ID 1071 [NCBI (National Center for Biotechnology Information) Entrez Gene database], nulceotide sequence NM_000078 (NCBI Entrez Nucleotide database) and − 1337C/T SNP rs17231506 (NCBI SNP database); and *LIPC*, gene ID 3990 (NCBI Entrez Gene database), nucleotide sequence NM_000236 (NCBI Entrez Nucleotide database), − 514 C/T SNP rs1800588 (NCBI SNP database) and − 514 C/T USF binding site ccttttgaca(c/t)gggggtgaag.

Table 1 Characteristics of subjects in this study

Values are means ± S.E.M. HDL-C* was adjusted by multiple linear regression analysis, including gender, alcohol intake, smoking and BMI.

Parameter	CAD	non-CAD	*P* value
Gender (male/female)	77/17	77/35	0.0303
Age (years)	52 ± 12	50 ± 12	0.3001
BMI (kg/m^2)	23.7 ± 3.0	23.9 ± 2.7	0.5000
Total cholesterol (nmol/l)	8.34 ± 1.74	8.37 ± 1.63	0.9131
Triacylglycerol (nmol/l)	1.64 ± 0.69	1.65 ± 0.80	0.8618
HDL-C (nmol/l)	1.04 ± 0.28	1.09 ± 0.34	0.2052
HDL-C* (mmol/l)	1.17 ± 0.28	1.22 ± 0.31	0.3888
LDL-C (nmol/l)	6.55 ± 1.79	6.53 ± 1.66	0.8658
ApoAI (g/l)	1.01 ± 0.25	1.08 ± 0.24	0.1222
ApoB (g/l)	1.77 ± 0.53	1.78 ± 0.44	0.9052
ApoE (g/l)	0.06 ± 0.03	0.06 ± 0.02	0.7330
Hypertension (*n*)	32 (34.0 %)	20 (17.9 %)	0.0070
Diabetes mellitus (*n*)	34 (36.2 %)	22 (19.6 %)	0.0079
Smokers (*n*)	54 (57.4 %)	63 (56.2 %)	0.8629
Alcohol drinkers (*n*)	36 (38.3 %)	44 (39.3 %)	0.8848
CSI	23.7 ± 7.4	5.8 ± 4.3	< 0.0001

Statistical analyses

All values are expressed as means ± S.D. unless otherwise noted. The allele frequency was estimated by gene counting. One-way ANOVA was performed, followed by multiple comparisons using Fisher's protected least significant difference. Serum HDL-C was adjusted by multiple linear regression analysis. The prevalence of patients with hypertension, diabetes mellitus, current and past smoking, and alcohol drinking were compared between different groups using a χ^2 test. A multiple logistic regression analysis was used to predict CAD from the genotype of polymorphism, with conventional risk factors as covariates. A probability value of $P < 0.05$ was considered to be significant. All tests were performed with StatView software (version 5.0; SAS Institute).

RESULTS

Characteristics of study subjects

The clinical and biochemical characteristics of the study population either with CAD or without CAD (non-CAD) are summarized in Table 1. A total of 94 the subjects with heterozygotes FH were suffering from CAD. There were significantly more males and subjects with hypertension and diabetes mellitus in the CAD group compared with the non-CAD group.

Association between − 1337 C > T polymorphism and CSI

The frequency of the *CETP* promoter − 1337 T allele was 0.20 in both males and females; lower than in Caucasians [12]. A few subjects in the present study had

Table 2 *CETP* − 1337 C > T polymorphism and plasma CETP levels

P value was determined using χ^2 test.

	CETP genotype				
	− 1337 CC		− 1337 CT + TT		
	n	CETP (μg/ml)	*n*	CETP (μg/ml)	*P* value
All	31	3.1 ± 1.1	13	2.6 ± 0.6	0.1364
Male	17	2.6 ± 0.6	8	2.4 ± 0.6	0.3139
Female	14	3.6 ± 1.3	5	3.0 ± 0.5	0.2831

the *CETP* promoter − 1337 TT genotype (11 males and two females), and the T allele was less frequent in subjects with a CSI ⩾ 14. The distribution of the *CETP* promoter − 1337 CC genotype differed significantly between those with a CSI ⩾ 14 and those with a CSI < 14 ($P = 0.0426$, as determined by a χ^2 test).

CETP promoter polymorphism and CETP concentrations

We compared plasma CETP concentrations between the − 1337 CC and − 1337 CT + TT genotypes in a subset of 44 subjects (25 males; Table 2). The CETP concentration tended to be lower in the presence of the T allele ($P = 0.14$).

CETP promoter polymorphism, lipid profile and development of CAD

The characteristics of subjects according to *CETP* promoter polymorphism are summarized in Table 3. As there were only two females with the TT genotype, we analysed men and women combined. HDL-C levels were not significantly higher in TT genotype, and the CSI tended to be lower in patients carrying the T allele ($P = 0.19$).

Effects of *CETP* and *LIPC* promoter polymorphisms on lipid profile and CSI

The frequency of the *LIPC* promoter − 514 T allele was 0.53 in males and 0.50 in females, which is similar to the frequencies previously reported in Japanese subjects, but higher than those in Caucasians [22,23]. To investigate the effects of *CETP* and *LIPC* promoter polymorphisms on lipid profile, we compared four subgroups stratified by high *CETP* genotype CC compared with low CETP CT + TT, and high *LIPC* genotype CC compared with low *LIPC* genotype CT + TT. Figure 1 shows that the HDL-C level was significantly higher in − 514 CC/− 1337 CT + TT than in − 514 CC/− 1337 CC [1.22 ± 0.36 mmol/l (47 ± 14 mg/dl) compared with 0.98 ± 0.30 mmol/l (38 ± 10 mg/dl) respectively; $P < 0.02$], and it was significantly higher in − 514 CC/− 1337 CT + TT than in − 514 CT + TT/− 1337 CC or in both CT + TT ($P < 0.05$). LDL-C levels did not differ significantly between the four groups. CSI was significantly lower in − 514 CC/− 1337 CT + TT than in − 514 CC/− 1337 CC (9.6 compared with 17.2 respectively; $P = 0.02$), suggesting an interaction between *CETP* and *LIPC* genotype on CSI.

Table 3 Characteristics of the subjects according to *CETP* genotype status

Values are means ± S.E.M. HDL-C* was adjusted by multiple linear regression analysis, including gender, alcohol intake, smoking and BMI.

	CETP genotype		
	CC	CT	TT
n	127	66	13
Total cholesterol (nmol/l)	8.50 ± 1.71	8.18 ± 1.66	7.87 ± 1.27
Triacylglycerol (nmol/l)	1.62 ± 0.72	1.73 ± 0.82	1.48 ± 0.57
HDL-C (nmol/l)	1.04 ± 0.28	1.09 ± 0.37	1.17 ± 0.37
HDL-C* (mmol/l)	1.19 ± 0.28	1.22 ± 0.37	1.23 ± 0.37
LDL-C (nmol/l)	6.71 ± 1.81	6.29 ± 1.61	6.03 ± 1.24
ApoAI (g/l)	1.02 ± 0.25	1.09 ± 0.25	1.08 ± 0.21
ApoB(g/l)	1.81 ± 0.50	1.76 ± 0.47	1.66 ± 0.36
ApoE(g/l)	0.07 ± 0.03	0.07 ± 0.03	0.05 ± 0.02
Age (years)	50 ± 11	53 ± 13	48 ± 11
BMI (kg/m^2)	23.7 ± 2.7	24.3 ± 3.2	22.8 ± 2.5
Smokers (%)	69 (54.3)	38 (57.6)	10 (76.9)
Hypertension (%)	30 (23.6)	18 (27.3)	4 (30.8)
Diabetes mellitus (%)	35 (27.6)	19 (28.8)	2 (15.4)
CSI	15.0 ± 10.7	12.4 ± 10.6	11.6 ± 9.6

Multiple logistic regression analysis

A multiple logistic regression analysis was performed to determine the association of CAD and *CETP* promoter polymorphism and other conventional risk factors. Gender, hypertension, diabetes mellitus and *CETP* − 1337 CC genotype exhibited significantly higher odds ratios; however, age, smoking, HDL-C and triacylglycerol levels, and the presence of *LIPC* − 514 C > T were not significant variates (Table 4).

DISCUSSION

The present study investigated the effects of *CETP* and *LIPC* promoter polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in subjects with heterozygous FH. None of the other coronary risk factors differed significantly between *CETP* genotypes; however, multiple logistic regression analysis revealed that coronary atherosclerosis was associated with the *CETP* − 1337 CC genotype. An interaction between the *CETP* and *LIPC* genotypes for plasma HDL-C and CAD has also been shown.

To our knowledge, this is the first study on the effects of the *CETP* promoter − 1337 C > T polymorphism

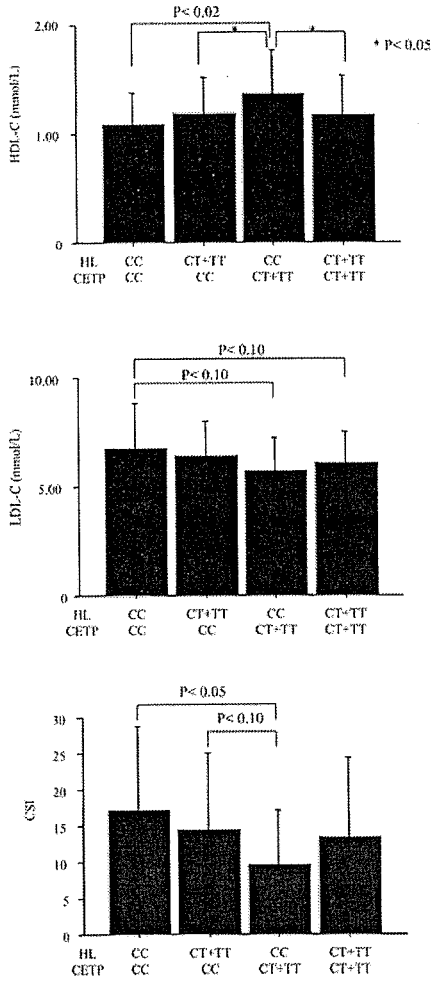

Figure 1 Effects of *CETP* and *LIPC* promoter polymorphisms on LDL-C, HDL-C and CSI

Subjects: −514 CC/−1337 CT+TT ($n=29$); −514 CT+TT/−1337 CC ($n=98$); −514 CC /−1337 CT+TT ($n=17$); −514 CT+TT/−1337 CT+TT ($n=62$). The HDL-C level was significantly higher in −514 CC/−1337 CT+TT than in −514 CC/−1337 CC (1.22 ± 0.36 compared with 0.98 ± 0.03 mmol/l; $P < 0.02$), and it was significantly higher in −514 CC/−1337 CT+TT than in −514 CT+TT/−1337 CC or CT+TT ($P < 0.05$). The LDL-C levels did not differ significantly between the four groups. CSI was significantly lower in −514 CC/−1337 CT+TT subjects than in −514 CC/−1337 CC subjects (9.6 compared with 17.2; $P = 0.02$).

in coronary atherosclerosis and, therefore, the first to suggest that the *CETP* promoter −1337 C>T polymorphism is associated with the severity of coronary atherosclerosis in heterozygous FH. In a previous study [8], this polymorphism was associated with low plasma CETP concentrations and high HDL-C levels more strongly than with the *Taq*IB2 allele in elderly Japanese males and, recently, this polymorphism has been reported to be a major determinant of promoter activity and plasma CETP concentration in REGRESS [12]. Therefore we

Table 4 Multivariate adjusted relative prevalence odds ratio of coronary atherosclerosis by multiple logistic regression analysis

For sex, male = 1 and female = 0; for hypertension, yes = 1 and no = 0; for diabetes mellitus, yes = 1 and no = 0; for *CETP* −1337 C>T polymorphism, CC = 1 and T+ = 0; for *LIPC* −514 C>T polymorphism, CC = 2, CT = 1 and TT = 0.

Variate	Odds ratio	*P* value
Age	1.021 (0.993–1.050)	0.1431
Sex	4.283 (1.788–10.259)	0.0011
Hypertension	2.628 (1.252–5.519)	0.0107
Diabetes mellitus	2.136 (1.081–4.218)	0.0289
Smoking	0.992 (0.969–1.015)	0.3261
CETP −1337 C>T polymorphism	2.022 (1.090–3.754)	0.0256
LIPC −514 C>T polymorphism	0.856 (0.562–1.305)	0.4698

investigated this −1337 site rather than the well-known *Taq*IB polymorphism. As subjects with FH have a high risk of premature CAD, we determined the existence of early stage coronary atherosclerotic changes by using CSI. Our present data suggest that the association of the *CETP* genotype with cardiovascular risk is independent of serum HDL-C levels. As indicated in Table 3, there was no significant difference in HDL-C/adjusted HDL-C levels between *CETP* genotypes. The *CETP Taq*IB2 allele was associated with HDL-C, especially HDL_2-C, in Japanese subjects [24] and, therefore, if we had assessed HDL_2-C, this might have revealed a significant difference between the genotypes.

There are conflicting reports as to whether CETP is pro- or anti-atherogenic. Humans with homozygous CETP deficiency have markedly high HDL-C levels and decreased LDL-C levels, with no clear evidence of premature atherosclerosis [2]. A *CETP* gene mutation (D442G) was shown to be associated with increased LDL particle size [25], suggesting that CETP is pro-atherogenic. In contrast, Hirano et al. [26] have reported that the prevalence of CETP deficiency was lower in individuals older than 80 years of age residing in a district of northern Japan, suggesting that CETP deficiency is not association with longevity, and the same investigators have shown that reduced CETP activity in conjunction with reduced HL activity is associated with an increased risk of CAD [27]. On the other hand, Moriyama et al. [28] found in a cross-sectional analysis that HDL-C elevation (⩾ 80 mg/dl) was protective against coronary heart disease, regardless of *CETP* genotype, in 19044 male and 29487 female Japanese subjects. In addition, a recent prospective study in the Honolulu Heart Program has shown the protective effects of heterozygous CETP deficiency against CAD, although the effect was not statistically significant [29].

At lower CETP concentrations, LDL-receptor activity is up-regulated, causing a reduction in serum LDL levels and leading to atheroprotection. Lowering CETP activity may be beneficial in an affluent environment, where high-fat and cholesterol-rich diets increase plasma LDL-C levels and down-regulate hepatic LDL-receptors, such as in FH. We presume that individuals with FH have higher CETP activity or concentration than normolipidaemic controls [30,31], which would be less pro-atherogenetic when they carry the *CETP* promoter −1337 T allele. De Grooth et al. [32] reported a significant positive correlation between carotid intima-media thickness and CETP levels in FH, suggesting that plasma CETP would be pro-atherogenic in FH. There are also some reports on the *CETP Taq*IB polymorphism and impaired glucose tolerance [33], suggesting that CETP could be pro-atherogenic independently of lipid metabolism. In the present study, however, there was no significant difference between *CETP* promoter −1337 C > T polymorphism and serum glucose levels (5.99 ± 1.94 mmol/l in −1337 CC compared with 5.72 ± 1.33 mmol/l in −1337 CT + TT; $P = 0.20$), and no difference in diabetes prevalence (results not shown).

In addition to CETP, HL also plays a crucial role in the metabolism of plasma lipoproteins, but the effects of CETP and HL activity on lipid profile and CAD are unclear [14,34]. The present study found no association between the *LIPC* promoter −514 C > T polymorphism and CAD and HDL-C levels; however, CSI with the *CETP* −1337 T allele and *LIPC* −514 CC was lowest in the subgroup. In another study from our laboratory (M. Takata and A. Inazu, unpublished work), HL activity was significant higher in −514 CC than CT + TT (0.282 ± 0.011 compared 0.231 ± 0.005 mmol/l respectively, $P < 0.001$) in hyperlipidaemic patients ($n = 325$, of which 183 were male). In human studies, HL activity tends to be elevated in the presence of smoking [35], insulin resistance in Type II diabetes mellitus [36], in females with omental fat mass [37] and males in general. These reports suggest that HL is pro-atherogenic. On the other hand, it has been reported that HL activity is lower in patients with CAD than in those without CAD [38]. Another group found that HL activity did not differ between subjects with and without CAD in REGRESS [39]. In an environment of low HL activity, IDL increases and it may be pro-atherogenic [40]. HL also promotes the formation of small and dense atherogenic LDL particles [13]. Lowering HL activity in hypertriglyceridaemia may decrease the pro-atherogenic risk due to an improved lipid profile, notably an increased LDL size [14]. In conditions where LDL-receptor activity is low, as in FH, HL activity appears to be inversely associated with CAD in subjects with low CETP concentrations (Figure 1), suggesting that the flux of cholesterol through the system of HDL-C transport may be more important in preventing atherosclerosis.

The main limitations of the present study were the relatively small sample size and the absence of data on HDL subclass and LDL particle size.

In conclusion, the *CETP* promoter −1337 C > T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels. We believe that this genetic variant of the *CETP* gene promoter could be an important determinant of coronary atherosclerosis in FH, and genotype differences between promoter variants and missense mutations need to be clarified in future investigations.

ACKNOWLEDGMENTS

We express special thanks to the lipidologists and cardiologists at the Second Department of Internal Medicine, Kanazawa University, and also to Sachio Yamamoto, Mihoko Mizuno and Mayumi Yoshida for their technical assistance. This work was supported by the Scientific Research Grant from the Ministry of Education, Science and Culture of Japan (no. 10770568 and 0907010).

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Received 20 April 2006/21 June 2006; accepted 6 July 2006
Published as Immediate Publication 6 July 2006, doi:10.1042/CS20060088

Cardiac Resurrection After Bone-Marrow-Derived Mononuclear Cell Transplantation During Left Ventricular Assist Device Support

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We describe a novel therapy of mononuclear cell transplantation combined with a left ventricular assist device (LVAD) for severe ischemic heart failure. Significant myocardial recovery by the LVAD rarely occurs in the severely failing heart. We undertook successful mononuclear cell transplantation in a patient who sustained an acute myocardial infarction that had resulted in the LVAD therapy. The heart regained good function after cell transplantation, and the LVAD was explanted 6 weeks later. These results suggest that this novel therapy could be an alternative to cardiac transplantation for severe ischemic heart failure.

(Ann Thorac Surg 2007;83:661–2)

The discovery of pluripotent stem cells in an adult has opened a novel clinical research field, regenerative medicine [1]. Many studies have demonstrated that bone-marrow-derived progenitor cells can differentiate into cardiomyocytes and endothelial cells, and they can be involved in repairing injured hearts [2]. Several clinical trials of autologous bone-marrow-derived mononuclear cell transplantation after acute myocardial infarction revealed the steady improvement in cardiac function [3]. We report a successful myocardial recovery with mononuclear cell transplantation and left ventricular assist device (LVAD) support after cardiogenic shock due to acute myocardial infarction.

A 61-year-old man who had diabetes mellitus was transferred in a shock state due to acute myocardial infarction. Emergency cardiac catheterization demonstrated the diagnosis of complete occlusion of the #7 left anterior descending artery (LAD) and 90% stenosis of the #2 right coronary artery (RCA). The culprit lesion, #7LAD, was not eligible for percutaneous coronary intervention because the wire could not cross it. Thereafter, the patient's shock state was worse, and percutaneous cardiopulmonary support was initiated.

Accepted for publication June 23, 2006.

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Despite maximum pharmacologic support, the patient lapsed into multiple organ failure. The decision was made implant a Toyobo LVAD (Toyobo, Inc, Osaka, Japan), and simultaneously perform a coronary artery bypass graft to the LAD and RCA with saphenous veins.

The status of multiple organ failure was gradually improved, but an ejection fraction (EF) by echocardiography was 0.13 on day 97 after LVAD implantation (Fig 1). Scintigraphy demonstrated complete infarctions in the anteroseptal and inferior walls (Fig 2). The patient was briefed in detail about mononuclear cell transplantation. This clinical study had been approved by the Ethics Committee of the Saitama Medical School, Saitama, Japan.

Bone marrow (600 mL) was aspirated under general anesthesia from both posterior ilia and enriched to the mononuclear cell fraction. The mononuclear cells were implanted in the infarcted zone through the saphenous grafts to the LAD and RCA on day 99 after LVAD implantation. During the procedure, the electrocardiogram was monitored and did not demonstrate any significant changes to suggest ischemic events. The possibility of microemboli was also negative on the basis of the stable normal values for creatine kinase-MB fraction and troponin-T after the procedure.

The patient's cardiac function became gradually better with time after the cell transplantation. The LVAD was removed on day 43 after mononuclear cell transplantation. The EF increased from 0.064 to .40 and remained stable, as assessed by echocardiography. Analysis of LV function by scintigraphy demonstrated a sustained improvement in blood perfusion and regional EF in the apical and inferior walls and growing thickness of the septal and inferior walls in the time course (Fig 2). The patient was discharged 58 days after explantation of the LVAD. After mononuclear cell transplantation, there were no complications, including acute inflammatory response, novel infarction, malignant arrhythmias, or ectopic differentiation.

Comment

Our aim was to extend the target of cell transplantation from the mild to the severely failing heart. The requirements were (1) a donor cell type, (2) an implantation procedure, including duration, route, and targets for cell delivery, and (3) a timing of cell transplantation after LVAD implantation. We chose bone-marrow-derived mononuclear cells to contain all of the cell fractions because (1) there is a controversy about the cell source to be grafted, (2) nobody can deny the possibility that the enrichment procedure will cause the useful cell population to be lost based on the current experimental data, and (3) pure, dense stem cells in a site might induce an ectopic differentiation.

As a grafting route, an antegrade intracoronary infusion through the saphenous vein grafts was chosen to avoid the isolated islet-like formation of grafted cells by direct injection into the myocardium, which could not effectively induce neogenesis of either cardiomyocytes or coronary capillaries.

The final important issue of the protocol was the timing of the cell transplantation after LVAD implantation. Although the LVAD improves cardiac milieu interne in the

Published by Elsevier Inc

0003-4975/07/$32.00
doi:10.1016/j.athoracsur.2006.06.074

Ann Thorac Surg
2007;83:661–2

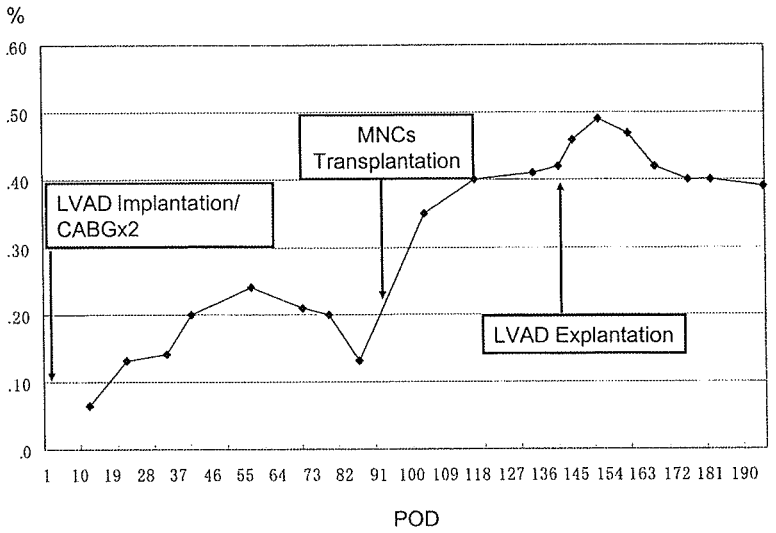

Fig 1. Left ventricular ejection fraction (EF) in transthoracic echocardiography. After left ventricular assist system (LVAS) implantation, the first evaluation showed an EF of 6.4%. In the time course under LVAS support, EF gradually improved, but the value began decreasing 2 months later. (POD = postoperative day; CABG = coronary artery bypass grafting; MNCs = mononuclear cells; LVAD = left ventricular assist device.)

FEATURE ARTICLES

early phase, long-term LVAD support induces ventricular atrophy so that the LVAD is a double-edged sword. We had been examining EF, LV wall thickness, and motions by echocardiography. Because the decline of EF commenced on day 72 after LVAD implantation, we judged that the global effects of LVAD for cardiac recovery had turned from benefits to drawbacks and performed mononuclear cell transplantation on day 99 after LVAD implantation.

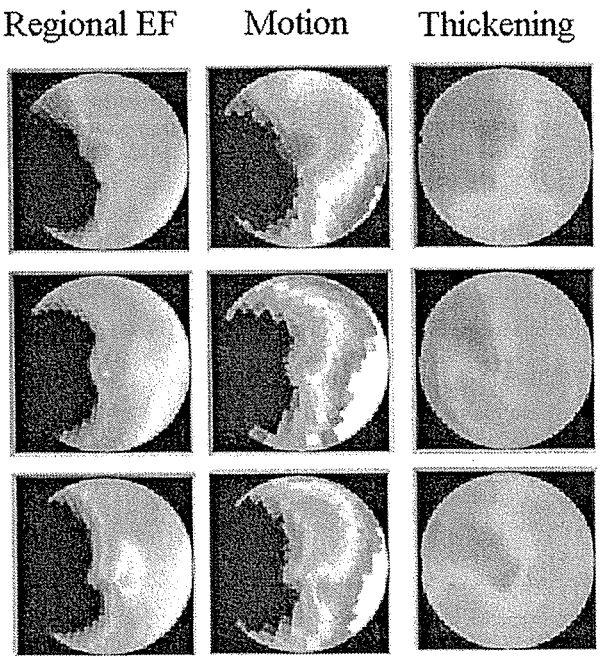

Fig 2. Technetium (Tc 99m)-tetrofosmin-gated single photon emission computed tomography. (Upper panels) Thirty-seven days after left ventricular assist device (LVAD) implantation and two coronary artery bypass grafts. (Middle panels) Twenty-nine days after mononuclear cell transplantation. (Lower panels) Twenty-five days after LVAD explantation (57 days after mononuclear cell transplantation).

The structural and functional improvements in last scintigraphy compared with that 1 month after mononuclear cell transplantation suggest that the cells engrafted, survived, and functioned in recipient heart. Grafted mononuclear cells release a wide array of cytokines related to the regeneration process. Mononuclear cell transplantation might stimulate the native environment to promote angiogenesis and cardiomyogenesis through the paracrine fashion in addition to vasculogenesis by the mononuclear cells themselves.

Many reports demonstrated that the LVAD support could induce reverse remodeling. In addition to relief of myocardium from the mechanical stretch of LVAD, the reverse remodeling of diseased heart could facilitate the engraftment, survival, and differentiation process of the grafted cells. Taken together, we think that mononuclear cell transplantation could fully work to repair the end-stage failing heart under the resting state created by LVAD support. This synergy effect of mononuclear cell transplantation and LVAD could be an explanation of this cardiac resurrection.

In conclusion, mononuclear cell transplantation for the treatment of ischemic cardiomyopathy with LVAD led to successful recovery of the failing heart and the LVAD to be unnecessary. We believe that this combination therapy might be an alternative to cardiac transplantation in the treatment of ischemic end-stage heart failure.

This work was supported in part by a Research Grant for Cardiovascular Diseases (16C-6) from the Ministry of Health, Labour and Welfare.

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Circ J 2006; **70:** 800–801

Stent Deformity Caused by Coronary Artery Spasm

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Previous studies have shown that coronary stents have radial strength above the pressure induced by coronary artery spasm. This case report describes a stent deformity caused by coronary artery spasm during percutaneous coronary intervention. (*Circ J* 2006; **70:** 800–801)

Key Words: Angioplasty; Stent; Vasospasm

Despite full medical treatment with calcium channel blockers and nitrates, some patients with coronary artery spasm continue to have recurrent episodes of angina and myocardial infarction, and arrhythmic sudden death might occur[1–6] Previous reports demonstrate the usefulness of coronary stents to prevent vasospasm refractory to medical therapy[7–9] However, coronary stent implantation is sometimes ineffective in patients with multivessel spasm[10] This case report describes another limitation of coronary stent implantation to prevent coronary artery spasm.

Case Report

A 53-year-old man had been well until he had severe chest pain caused by acute myocardial infarction. He was referred 2 weeks after the onset of acute myocardial infarction. Coronary angiography revealed a 90% stenosis in the proximal obtuse marginal artery (Fig 1A). There was no significant stenosis in the left anterior descending coronary artery and right coronary artery. A 0.014-inch Skipper guidewire (Asahi Intecc, Nagoya, Japan) was placed across the lesion into the distal obtuse marginal artery. Predilation was performed by using a 3.0-mm OMNIPASS balloon catheter (Cordis, Miami, FL, USA) inflated to 6 atm. Intravascular ultrasound (IVUS) imaging was performed in the obtuse marginal artery using a 30-MHz 3.2F Ultracross catheter (Boston Scientific, Natick, MA, USA). The IVUS image showed a significant stenosis with fibrofatty plaque. A 25 mm NIR stent premounted on a 3.0-mm balloon catheter (Medinol, Tel Aviv, Israel) was deployed in the proximal obtuse marginal artery using an inflation pressure of 14 atm. Angiography and IVUS showed a good result (Figs 1B,2A). After the guidewire was withdrawn, the patient complained of chest pain; electrocardiogram demonstrated ST-segment elevation in lead I and aVL and the systolic blood pressure dropped from 140 to 80 mmHg. Angiography demonstrated coronary artery spasm at the proximal stented segment and distal reference (Fig 1C). Thereafter, stent deformity was observed (Fig 2B). Intravenous norepinephrine (0.2 mg) and intracoronary nitroglycerine (200 μg) were administered. The systolic blood pressure increased to 100 mm Hg and the coronary artery spasm was relieved (Fig 1D). Further coronary intervention was not performed because there was no flow disturbance (TIMI 3) in the obtuse marginal artery. The patient received oral aspirin (100 mg daily), ticlopidine (100 mg twice daily for 4 weeks), diltiazem (100 mg daily), and isosorbide mononitrate (20 mg twice daily). There was no in-hospital event. During follow-up, no adverse event was observed. Six months later, follow-up angiography was performed. The deformed stent (Fig 2C) and a 25% stenosis at the distal stented segment (Fig 1E) were observed.

(Received January 10, 2006; revised manuscript received March 10, 2006; accepted March 28, 2006)

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Discussion

Calcium antagonists and nitrates are effective in preventing coronary artery spasm in most cases. However, coronary artery spasm refractory to the treatment with these drugs is observed in some cases[1–6] Alpha-1 blocking agents[2] magnesium[3] benzhexol hydrochloride[4] denopamine[5] and nicorandil[6] have been reported as alternatives. Previous reports demonstrated the efficacy of coronary stenting in patients with clinically severe coronary artery spasm refractory to aggressive pharmacologic management[7–9] Gaspardone et al evaluated the usefulness of coronary stent placement in 9 patients with vasospastic angina refractory to medical treatment[10] The NIR stent was used in 6 patients. During follow-up, 3 patients developed recurrent episodes of angina at rest. Holter monitoring demonstrated ST-segment elevation associated with angina. Repeat coronary angiography showed coronary artery spasm after the administration of methylergometrine in these patients. Coronary artery spasm occurred proximally to the previously implanted stent in 2 patients and in other coronary arteries in 1 patient.

Coronary artery spasm is sometimes observed during percutaneous coronary intervention. It is usually relieved by the intracoronary administration of nitroglycerin. Balloon inflation at a low pressure may be used to treat it. Agrawal et al calculated the minimum acceptable collapse pressure for stents using arterial strain caused by experimentally induced artery spasm[11] They reported 0.4 atm as the minimum acceptable limit for collapse pressure. Almost all coronary stents have more radial strength[12,13] The NIR stent is one of the stents with strong radial strength[12] An in-vitro study reported that the NIR stent expanded to 3 mm

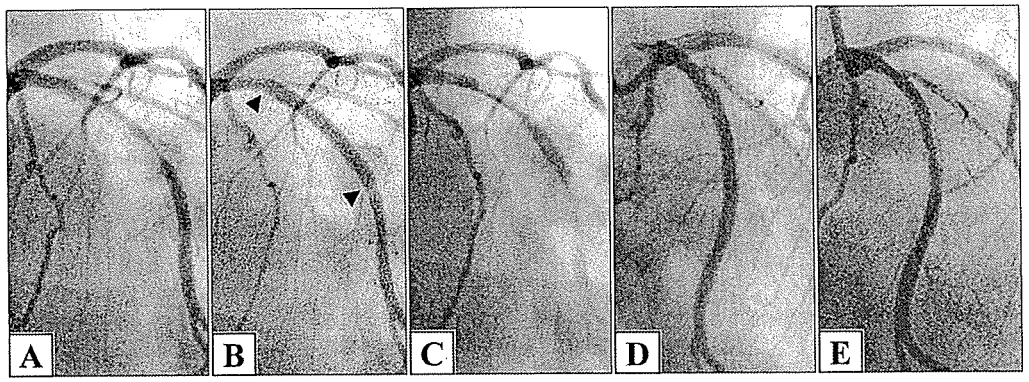

Fig 1. Coronary angiography showing a 90% stenosis in the proximal obtuse marginal artery (A). After deployment of a NIR stent, angiography demonstrates a good result (B). Arrowheads indicate the edges of the stent. Angiography demonstrates coronary artery spasm at the proximal stented segment and distal reference (C). After intracoronary administration of nitroglycerine, coronary artery spasm is relieved (D). Follow-up angiography shows a 25% stenosis at the distal stented segment (E).

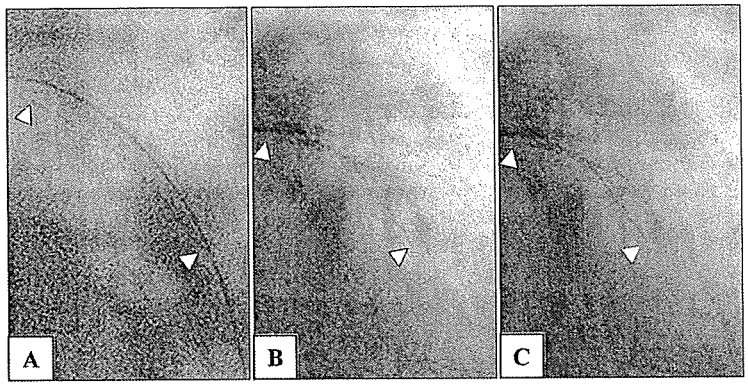

Fig 2. Fluoroscopy demonstrates a fully expanded stent (A). After coronary artery spasm, stent deformity is observed (B). Fluoroscopy shows the deformed stent at follow up (C). Arrowheads indicate the edges of the stent.

collapsed at a compressive strength of 1.05 atm.[12] This case report demonstrates unusually severe coronary artery spasm because the NIR stent was deformed. Coronary stenting may be ineffective in some patients with severe coronary artery spasm as well as in those with multivessel spasm. These are the limitations of stent implantation for coronary artery spasm. Thus, alternative medical treatment such as α-1 blocking agents,[2] magnesium,[3] benzhexol hydrochloride,[4] denopamine,[5] and nicorandil[6] should be tried for coronary artery spasm refractory to calcium antagonists and nitrates before stent implantation is considered. Stent implantation would be the last resort. In some patients, stent implantation for refractory coronary artery spasm might be performed. However, intensive medical treatment should be continued in those patients even after stent implantation.

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