Association of Serum MCP-1 Concentration and *MCP-1* Polymorphism with Insulin Resistance in Japanese Individuals with Obese type 2 Diabetes

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  Monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) secreted by adipocytes is a member of the CC chemokine family and plays a pivotal role in the inflammatory process. A polymorphism, the −2518 A/G of *MCP-1* gene, has been associated with type 2 diabetes, type 1 diabetes, parameters of insulin resistance and obesity. Therefore, we investigated the effects of *MCP-1* single nucleotide polymorphisms (SNPs) on the susceptibility to type 2 diabetes or insulin resistance in the Japanese population. We also assessed the correlation between serum MCP-1 concentration and other clinical characteristics in Japanese type 2 diabetic subjects. The serum MCP-1 concentration was significantly correlated with HOMA-IR and the visceral fat area, but not with BMI. Although there was no association between this SNP and type 2 diabetes, the -2518A/G polymorphism was associated with the serum MCP-1 concentration. In subgroup analysis, Japanese obese diabetic -2518AA carriers had a higher MCP-1 concentration and increased insulin resistance than obese diabetic -2518G carriers. These data indicated that the *MCP-1* polymorphism was associated with insulin resistance in Japanese obese diabetic subjects and that *MCP-1* was implicated in the pathogenesis of insulin resistance, especially associated with obesity, in humans.

  Type 2 diabetes mellitus is a complex disease characterized by impairments of both insulin secretion and insulin resistance. From 60% to 90% of type 2 diabetes appears to be related to obesity or weight gain [1]. Insulin resistance is a characteristic feature of type 2 diabetes associated with obesity or metabolic syndrome. Adipocytokines, including leptin, adiponectin, retinol-binding protein 4 (RBP4), and MCP-1, are secreted by adipocytes and seem to be implicated in the pathogenesis of insulin resistance associated with obesity [11].

  MCP-1 is a member of the CC chemokine family and plays a critical role in recruiting monocytes, memory T cells, and basophils, and in atherogenesis [3,28]. Deletion of *MCP-1* in LDL receptor-null mice attenuated the severity of atherosclerotic disease [8]. Recently, Kamei et al. and we reported that mice expressing an *MCP-1* transgene in adipose tissue exhibited insulin resistance, macrophage infiltration into adipose tissue, and increased hepatic triglyceride content [9,10]. *MCP-1* gene expression in adipose tissue of an obese mouse model was increased [9,10,21] and mice lacking *MCP-1* manifested decreased insulin

resistance, macrophage infiltration into adipose tissue and hepatic steatosis [10]. Recent studies in humans reported positive correlations between the serum MCP-1 level and HOMA-IR [13] or body mass index (BMI) [13,18]. A polymorphism, −2518 A/G, was originally reported to affect *MCP-1* transcription activity [20]. Subsequently, this polymorphism has been associated with type 2 diabetes, insulin resistance [22], type 1 diabetes [27], and cardiovascular disease [23]; however, these associations are still controversial. On the basis of our recent observations in mice [10], we screened variation(s) in the *MCP-1* gene, which would have an effect on the susceptibility to type 2 diabetes or on clinical characteristics such as insulin resistance or fat distribution.

SUBJECTS AND METHODS

Subjects

A total of 763 patients with type 2 diabetes and 361 nondiabetic individuals were enrolled in the study. All subjects were Japanese. Diabetes was diagnosed according to the criteria of the American Diabetes Association (1997), and the nondiabetic subjects were selected according to no past history of diabetes mellitus or impaired glucose tolerance. A total of 104 diabetic patients (60 men, 44 women), who had not been treated with insulin, were evaluated for their clinical profiles. The study was performed with written informed consent from all subjects and was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

Clinical assessment

The BMI of each individual was directly measured at the time of collecting blood samples. The fasting plasma glucose concentration (FPG), fasting serum immunoreactive insulin concentration (FIRI), serum lipid concentration, and HbA_{1c} level were determined by standard laboratory techniques calibrated with uniform standards. Serum MCP-1 concentrations were measured by immunoassay system, Quantikine human MCP-1 Immunoassay (R&D Systems Inc., Minneapolis, MN). The homeostasis model assessment for insulin resistance (HOMA-IR) was used as an index of insulin resistance, calculated as [FPG (mmol/l) × FIRI (μU/mL)] / 22.5 [17]. The subcutaneous fat area, visceral fat area, hepatic lipid content (HLC) and intramyocellular lipid content (IMCL) were determined by nuclear magnetic resonance as described previously [6,12]. We also assessed subjects in subgroups: obese diabetic subgroup (BMI ≥ $25kg/m^2$, n = 59) and non-obese diabetic subgroup (BMI<$25kg/m^2$, n = 45). Clinical characteristics are shown in Tables 1 and 2.

Hyperinsulinemic-euglycemic clamp

Regular human insulin was infused intravenously at 1.46 mU/kg/min to achieve a serum insulin concentration of 100 μU/mL, and plasma glucose concentration was maintained at 5.5 mmol/L by a variable infusion of glucose. The hyperinsulinemic-euglycemic clamp was maintained for at least 2 hours. The rate of glucose infusion (GIR), expressed in milligrams per kilogram per minute, required to maintain euglycemia during hyperinsulinemia was used as an estimate of insulin-stimulated total-body glucose uptake [4,14].

Sequencing of MCP-1 and genotyping of SNPs

Genomic DNA was extracted from blood with a QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). Specific oligonucleotide primer sets were designed on the basis of MCP-1 sequence information in GenBank (accession numbers NT_010799 and NM_002982). All three coding exons (including exon-intron boundaries) of *MCP-1* and 5′ flanking distal and proximal (-2650 ~ -2154 and -493　bp, relative to transcription starting site of NM_002982, respectively) regulatory regions were amplified by polymerase chain reaction (PCR) and were resequenced for 128 Japanese diabetic subjects. PCR was carried

out using Ex Taq Polymerase (TaKaRa Biotechnology, Shiga, Japan). Sequencing was carried out using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) on an automated DNA capillary sequencer (model 3100, Applied Biosystems). Sequence information about the primers and PCR conditions is shown in Table 3. SNP genotypes were determined by direct sequencing of PCR or a fluorescence- or colorimetry-based allele-specific DNA primer probe assay system (ASP-PCR methods; Toyobo Gene Analysis, Tsuruga, Japan) as described previously [26].

Table 1. Clinical characteristics of type 2 diabetic subjects and nondiabetic control subjects.

Characteristic	Type 2 diabetic subjects ($n = 763$)	Nondiabetic control subjects ($n = 361$)
Age (years)	63.0 ± 10.0	75.3 ± 8.0
BMI (kg/m^2)	23.6 ± 3.3	21.5 ± 3.8
HbA$_{1c}$ (%)	7.8 ± 1.7	5.1 ± 0.4
HOMA-IR	3.1 ± 3.5	N.A.
Total cholesterol (mg/dL)	202.6 ± 39.9	N.A.
Triglyceride (mg/dL)	141.0 ± 124.0	N.A.

Data are the means ± SD. Data of HOMA-IR from subjects who had not been treated with insulin. N.A.: not available.

Table 2. Clinical characteristics of type 2 diabetic subjects and obese type 2 diabetic subjects in the clinical assessment and the subgroup analysis.

Characteristic	Type 2 diabetic subjects ($n = 104$)	Obese diabetic subgroup ($n = 59$)
Age (years)	58.7 ± 12.6	56.5 ± 13.4
BMI (kg/m^2)	25.8 ± 4.3	28.5 ± 3.3
HbA$_{1c}$ (%)	8.9 ± 2.6	8.4 ± 1.8
HOMA-IR	3.3 ± 4.9	4.3 ± 6.3
Total cholesterol (mg/dL)	207.5 ± 43.6	213.2 ± 42.3
Triglyceride (mg/dL)	155.0 ± 74.6	166.1 ± 86.2
GIR (mg/kg/min)	5.8 ± 2.2	5.1 ± 1.7
MCP-1 (pg/mL)	302.7 ± 102.4	317.4 ± 111.7
SF (cm^2)	153.8 ± 73.3	186.6 ± 74.5
VF (cm^2)	113.8 ± 48.8	129.3 ± 52.3
IMCL (arbitrary units)	8.7 ± 4.1	9.3 ± 4.7
HLC (arbitrary units)	9.6 ± 6.3	11.2 ± 6.7

Data are the means ± SD. Obese diabetic subgroup: BMI $\geq$ 25kg/m^2. GIR: glucose infusion rate during the hyperinsulinemic-euglycemic clamp. SF: subcutaneous fat area. VF: visceral fat area. IMCL: intramyocellular lipid content. HLC: hepatic lipid content. Total cholesterol, triglyceride and MCP-1 were measured in serum.

Table 3. Sequences of primers and conditions for PCR amplification of *MCP-1*.

Primer Set	Forward primer sequence	Reverse primer sequence	Size (bp)	Temp (°C)	Polymerase
MCP-1 -2518	attctcttctacgggatctg	agctgaaagatgctgagatc	497	60	Ex Taq
MCP-1 prox	tctgctaggcttctatgatg	tggatgtttctgggttagtc	530	58	Ex Taq
MCP-1 ex1	ccacttatcactcatggaag	tggtccatgataactcagag	301	58	Ex Taq
MCP-1 ex2	cagaataatccagttcatcc	taaatagtgcactctctgac	267	56	Ex Taq
MCP-1 ex3	tctagaccaaaactgcaaag	agctcatactctcagctttg	588	57	Ex Taq

Size and temp refer to the size of PCR products and the annealing temperature, respectively. Specific oligonucleotide primers were designed on the basis of *MCP-1* sequence information in GenBank (accession number NT_010799). Genomic DNA (50 ng) was amplified in a 20-µL reaction mixture containing 0.5 U of Ex Taq polymerase (TaKaRa, Shiga, Japan), 10 pmol of each primer, polymerase buffer, and deoxynucleoside triphosphates. The amplification protocol comprised initial incubation at 94°C for 5 min and 30 cycles of 94°C for 30 s, the indicated annealing temperature for 30 s, and 72°C for 30 s; and a final incubation at 72°C for 5 min.

Statistical analysis

Averaged data are presented as the means ± SD. We assessed association and Hardy-Weinberg equilibrium by the chi-square test. Linkage disequilibrium (LD) and haplotype analyses were performed with SNPAlyze software version 5.1 Pro (Dynacom, Chiba, Japan). Haplotype estimation was performed by the expectation-maximization algorithm [5]. To measure LD between SNPs, we calculated D' for 128 diabetic individuals. Statistical analysis was performed with the Stat-View program (version 5.0-J; SAS Institute, Cary, NC, USA). Differences between groups were analyzed by the Mann-Whitney *U* test. Categorical variables were compared by chi-square test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Correlation between serum MCP-1 concentration and other clinical characteristics

We examined the correlations between serum MCP-1 concentration and various clinical characteristics in 104 diabetic patients. As shown in Table 4, the serum MCP-1 concentration was significantly correlated with HOMA-IR (r= 0.300, p = 0.002) and the visceral fat area (r= 0.312, p = 0.002), but not with BMI (r= 0.106, p = 0.291). GIR during the hyperinsulinemic-euglycemic clamp, subcutaneous fat area, IMCL and HLC were not associated with the serum MCP-1 level in type 2 diabetic subjects. In the subgroup analysis, we found a stronger correlation of serum MCP-1 concentration with HOMA-IR in the obese diabetic subgroup (obese diabetic subgroup: r= 0.347, non-obese diabetic subgroup: r= 0.122, data not shown).

Association between the MCP-1 -2518A/G polymorphism and type 2 diabetes

We identified six SNPs in 128 Japanese diabetic subjects, none of which was nonsynonymous. After excluding two SNPs (-2515A/G, -2348G/C) according to their minor allele frequencies < 5 %, we analyzed linkage disequilibrium (LD) using the remaining four SNPs, -2518A/G, -299C/G, +964C/T and +1606T/C (relative to the transcription starting site defined by Ueda et al. [25] according to the original report [11]). All SNPs were in a strong LD block (Figure 1, Table 5) and fulfilled Hardy-Weinberg equilibrium expectations. Given that these four SNPs were in a high LD block and -2518A/G polymorphism was reported to

be associated with type 2 diabetes in Caucasians, we focused on the -2518A/G polymorphism in the distal regulatory region. This SNP was genotyped in 1124 samples (763 type 2 diabetic subjects and 361 nondiabetic control subjects); however, there was no association between this SNP and the susceptibility to type 2 diabetes (Table 5). We also found that the frequency of a minor allele, -2518A, was 0.353 in Japanese nondiabetic control subjects. On the other hand, the -2518A allele was a major allele in Caucasians and its frequency was 0.64-0.73 [11-14].

Table 4. Correlation between serum MCP-1 level and clinical characteristics.

Characteristic	Type 2 diabetes (n = 104)		Obese diabetic subgroup (n = 59)	
	r	*P*	r	*P*
BMI	0.106	0.291	-0.173	0.200
HOMA-IR	**0.300**	**0.002**	**0.347**	**0.008**
GIR	-0.064	0.530	-0.157	0.258
Subcutaneous fat area	0.157	0.118	0.085	0.537
Visceral fat area	**0.312**	**0.002**	0.262	0.050
Intramyocellular lipid content	0.079	0.436	0.179	0.192
Hepatic lipid content	0.153	0.129	-0.089	0.514

Obese diabetic subgroup: BMI $\geq 25 kg/m^2$.
Correlations between serum MCP-1 levels and clinical characteristics were calculated by simple linear regression. Bold type: *P*<0.05.

Table 5. Association analysis for SNPs of *MCP-1* and type 2 diabetes.

Position	dbSNP ID	Major allele	Minor allele	n		MAF		OR (95% C.I.)	*P*
				Case	Control	Case	Control		
-2518	rs1024611	G	A	763	361	0.353	0.346	1.03 (0.86-1.24)	0.75
-2515	New	A	G	128		0.008			
-2348	rs3917903	G	C	128		0.043			
-299	rs2857656	C	G	128		0.289			
+964	rs4586	C	T	128		0.289			
+1606	rs13900	T	C	128		0.289			

Position is relative to the transcription starting site reported by Ueda et al. MAF: minor allele frequency. OR: odds ratio. *P* value was calculated by the chi-square test with allele data.
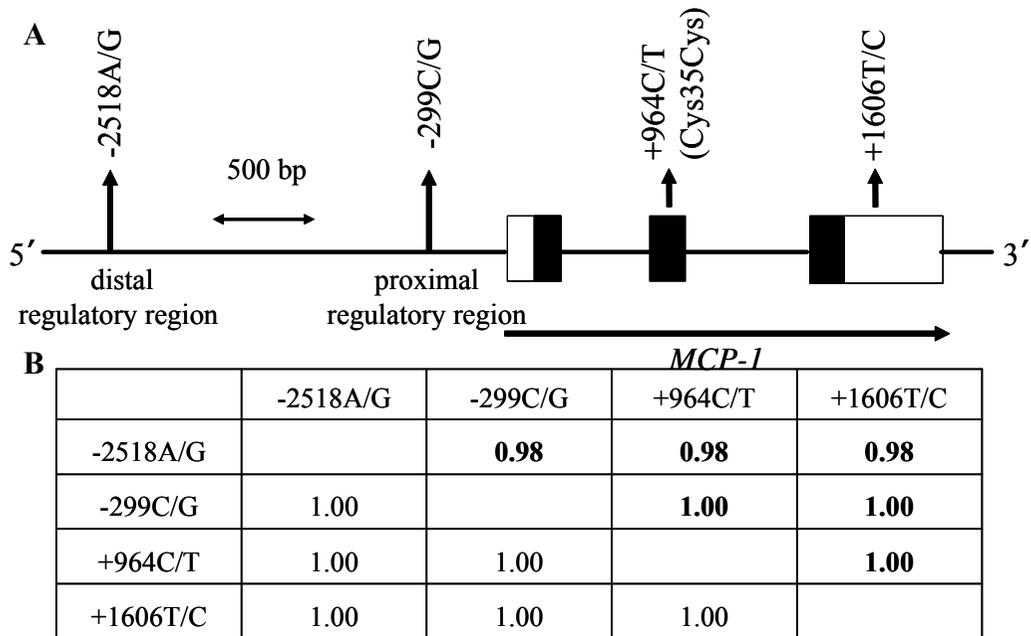
A

B

	-2518A/G	-299C/G	+964C/T	+1606T/C
-2518A/G		**0.98**	**0.98**	**0.98**
-299C/G	1.00		**1.00**	**1.00**
+964C/T	1.00	1.00		**1.00**
+1606T/C	1.00	1.00	1.00	

Figure 1. *MCP-1* gene structure and pairwise LD analysis of SNPs. (**A**) Schematic representation of *MCP-1* and SNP locations. Coding and noncoding sequences of exons are shown as closed and open boxes, respectively. (**B**) Values of *D'* (non-bold type) and of r^2 (bold type) for pairwise LD analysis. All SNPs are in a strong LD block.

Table 6. Clinical characteristics of type 2 diabetic subjects according to -2518A/G genotype for *MCP-1*.

Characteristic	Type 2 diabetes			Obese diabetic subgroup		
	GG+AG (*n* = 93)	AA (*n* = 11)	*P*	GG+AG (*n* = 51)	AA (*n* = 8)	*P*
BMI (kg/m^2)	25.7 ± 3.9	26.9 ± 6.6	0.16	28.4 ± 3.2	29.7 ± 4.3	0.19
HOMA-IR	3.3 ± 5.2	3.5 ± 2.5	0.46	3.4 ± 3.3	4.5 ± 2.5	0.11
Total cholesterol (mg/dL)	208 ± 45	207 ± 30	0.97	214 ± 43	206 ± 36	0.69
Triglyceride (mg/dL)	157 ± 77	141 ± 54	0.80	168 ± 91	153 ± 50	0.95
GIR (mg/kg/min)	5.8 ± 2.2	5.5 ± 2.7	0.37	**5.25 ± 1.71**	**3.96 ± 0.78**	**0.025**
MCP-1 (pg/mL)	**294 ± 98**	**373 ± 119**	**0.038**	**305 ± 107**	**396 ± 118**	**0.045**
SF (cm^2)	150 ± 69	185 ± 101	0.19	180 ± 73	226 ± 78	0.13
VF (cm^2)	112 ± 47	129 ± 62	0.3	125 ± 53	157 ± 45	0.08
IMCL (arbitrary units)	8.7 ± 4.2	9.1 ± 3.3	0.42	9.2 ± 4.9	9.9 ± 3.4	0.39
HLC (arbitrary units)	9.4 ± 6.3	11.4 ± 5.5	0.16	10.9 ± 7.0	13.1 ± 5.1	0.22

Data are the means ± SD. *P* values were calculated by the Mann-Whitney *U* test. Obese diabetic subgroup: BMI ≥ 25kg/m^2. GIR: glucose infusion rate during the hyperinsulinemic-euglycemic clamp. SF: subcutaneous fat area. VF: visceral fat area. IMCL: intramyocellular lipid content. HLC: hepatic lipid content. Total cholesterol, triglyceride and MCP-1 were measured in the serum. Bold type: *P*<0.05.

Association between the MCP-1 -2518A/G polymorphism and clinical characteristics

Next, we analyzed the possible effects of the -2518A/G polymorphism on clinical characteristics in 104 type 2 diabetic subjects. As shown in Table 6, the -2518A/G polymorphism was associated with serum MCP-1 concentration (GG+AG 294 ± 98 pg/mL vs AA 373 ± 119 pg/mL, p = 0.038). In the obese diabetic subgroup, the -2518A/G polymorphism was associated with serum MCP-1 concentration (GG+AG 305 ± 107 pg/mL vs AA 396 ± 118 pg/mL, p = 0.045) and GIR (GG+AG 5.25 ± 1.71 mg/kg/min vs AA 3.96 ± 0.78 mg/kg/min, p = 0.025). This observation indicated that obese diabetic -2518AA carriers had   higher serum MCP-1 concentration and increased insulin resistance compared with obese diabetic -2518G carriers in Japanese.

DISCUSSION

In this study, we found that the serum MCP-1 concentration was significantly correlated with the visceral fat area, but not with BMI in Japanese diabetic subjects. This finding was consistent with a previous Italian report in nondiabetic obese women [15]. MCP-1 release was higher in visceral adipose tissue compared with subcutaneous adipose tissue in the study of whole human adipose tissue cultures [2]. Insulin resistance and hepatic steatosis induced by a high-fat diet were reduced in MCP-1 KO mice [10]. Given these reports, MCP-1 released by visceral fat could affect predominantly on hepatic insulin sensitivity associated with obesity. In fact, we observed a significant correlation between the serum MCP-1 concentration and HOMA-IR, which was correlated well with hepatic insulin sensitivity measured with [^{3}H] glucose [16, 24]. This correlation was stronger in the Japanese obese diabetic subgroup than in the non-obese diabetic subgroup. Previously, the serum MCP-1 concentration was correlated with HOMA-IR in obese Korean subjects [13] or with QUICKI in Italian subjects [19]. Our data suggested that the circulating MCP-1, which may be mainly released by visceral fat, was implicated in the pathogenesis of insulin resistance, especially in the liver.

We showed that *MCP-1* -2518AA carriers have a higher serum MCP-1 concentration in Japanese obese diabetic subjects. Our observation suggested that the *MCP-1* -2518A allele might increase the MCP-1 secretion from visceral fat. This observation was consistent with a previous report showing that the *MCP-1* -2518A allele was associated with higher plasma MCP-1 concentration in Caucasians [22].and Meanwhile, others reported inconsistent observations [7,20]. The reason for this discrepancy was unknown. The *MCP-1* -2518A/G polymorphism also has been associated with type 2 diabetes [22] or type 1 diabetes [27]. We failed to detect an association between this polymorphism and type 2 diabetes in the Japanese population. Regarding the effect of this polymorphism on the susceptibility to diabetes or the serum concentration of MCP-1, further studies will be required in various ethnic groups.

ACKNOWLEDGMENTS

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