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Haplotype analysis of the human collectin placenta 1 (*hCL-P1*) gene

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Abstract Collectins are a family of C-type lectins found in vertebrates. These proteins have four regions, a relatively short N-terminal region, a collagen-like region, an alpha-helical coiled coil, and a carbohydrate recognition domain. Collectins are involved in host defense through their ability to bind carbohydrate antigens on microorganisms. Type A scavenger receptors are classical-type scavenger receptors that also have collagen-like domains. We previously described a new scavenger receptor, collectin from placenta [collectin placenta 1 (CL-P1)]. CL-P1 is a type II membrane protein with all four regions. We found that CL-P1 can bind and phagocytize both bacteria and yeast. In addition to that, it reacts with oxidized low-density lipoprotein (LDL) but not with acetylated LDL. These results suggest that CL-P1 might play important roles in host defenses and/or atherosclerosis formation. One rational strategy to study the role of CL-P1 in these pathological conditions would be to perform a haplotype association study using human samples. As a first step for this strategy, we analyzed the haplotype structure of the *CL-P1* gene. By sequencing the *CL-P1* gene in ten Japanese volunteers, we identified five single-nucleotide polymorphisms (SNPs) with a minor allele frequency of at least 29%. To obtain SNPs in the 5'-upstream region of the gene, we screened a total of 20 SNPs described

in the database and finally picked up one SNP for the present study. Thus, a total of six SNPs, one in the 5'-upstream region, two in intron 2, one in exon 5, and two in exon 6, were used to analyze the haplotype structure of the gene, with DNAs derived from 54 individuals (108 alleles). The analysis revealed that only two of six SNPs showed significant linkage disequilibrium ($r^2 > 0.5$) with each other. This haplotype information may be useful in disease-association studies in which a contribution of the *CL-P1* gene has been suspected, especially in immunological disturbance or atherosclerosis. Two SNPs in exon 6, both leading to amino acid substitutions, could be candidates for influencing disease susceptibility.

Key words Single-nucleotide polymorphisms · Linkage disequilibrium · Haplotype · Collectin · Japanese population · Host defense · Amino acid substitution · Atherosclerosis

Introduction

Collectins are a family of proteins that contain a collagen-like region and a carbohydrate recognition domain (CRD) (Drickamer 1988). Collectins are found in vertebrates including avians (Laursen et al. 1998). There are four groups of collectins: a mannan-binding protein (MBP) group, two lung surfactant groups of surfactant protein A (White et al. 1985) and surfactant protein D (SP-D) (Persson et al. 1989), and a newly isolated group, collectin liver 1 (Ohtani et al. 1999) and collectin placenta 1 (CL-P1) (Ohtani et al. 2001). Collectins are known to be involved in innate immunity through various mechanisms, for example, by activation of the complement pathway (Kawasaki et al. 1989), by opsonization via collectin receptors (Schweinle et al. 1989; van Iwaarden et al. 1992), and by amplification of phagocytosis by macrophages (Pikaar et al. 1995). Interestingly, the type A scavenger receptor (SR-A) also contains a collagen-like domain (Kodama et al. 1990), which could bind and destruct pathogens by endocytosis and phagocytosis. In fact, recent data with SR-A1 knock-out mice (Suzuki et al.

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1997) support the possibility that scavenger receptors also have a role in innate immunity.

Recently, we cloned and characterized the human *CL-P1* gene (*hCL-P1*), and found that it was a membrane-type collectin that functions as a scavenger receptor. The major tissue expression of the gene was endothelial cells but not in monocyte-macrophage lineage cells. We also found that CL-P1 was able to bind and phagocytose bacteria and yeast as well as oxidized low-density lipoprotein (Ohtani et al. 2001). With these results, we hypothesized that this gene could play an important role in the human innate immunity system and/or atherosclerosis formation. To study this possibility, we are collecting human samples that are susceptible to infection, especially in childhood, and also samples from patients with atherosclerosis without lipid profile abnormalities. Haplotype association studies would be the best choice for this purpose. Here, we report the haplotype structure of the *CL-P1* gene obtained from six single-nucleotide polymorphisms (SNPs) that were genotyped with 108 alleles in Japanese subjects.

Subjects and methods

Identification of SNPs

To identify useful SNPs in the gene region, we sequenced amplified DNAs derived from ten Japanese volunteers (20 alleles). Polymerase chain reaction (PCR) primers were chosen at approximately 500-bp intervals, with about an 80-bp overlap (Shinohara et al. 2001). A total of 16 primer sets were designed based on the information obtained from a public genome database (AC022545, AP000925, AP001005, <http://www.ncbi.nlm.nih.gov/>) to amplify all 10 exons; 5'-flanking regions; and introns 1, 4, 6, 7, and 9. Other introns were sequenced in the regions near exon-intron boundaries. After amplification, a sequencing reaction was performed and analyzed on an ABI 310 genetic analyzer (PE Biosystems, Foster City, CA, USA). SNPs were detected by alignment of the sequencing data of ten individuals. To obtain SNPs in the 5'-upstream region, we analyzed 20 SNPs described in the publicly available SNP database of The SNP Consortium Ltd (TSC; <http://snp.cshl.org/index.html>) with 20 Japanese alleles.

SNP genotyping

SNPs were genotyped using three PCR-based methods: single-strand conformational polymorphism (SSCP), SNaPshot, and sequencing. PCR was performed in a volume of 12.5 µl containing 20 ng genomic DNA, 75 mM Tris HCL (pH 8.8), 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 1.5 mM MgCl₂, 200 µM deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer, and 0.25 units of Taq polymerase. PCR amplification was performed through 30 cycles of 94°C for 30 s, 49–62°C for 30 s, and 72°C for 30 s, depending on the region analyzed, with a final extension step of 5 min at 72°C in a Gene Amp PCR9700 System (PE

Biosystems). For SSCP analysis, each 2.5 µl of PCR products were mixed with formamide dye and heat denatured, and then applied to polyacrylamide gel electrophoresis. After electrophoresis, the gel was silver stained (Wako Pure Chemical, Tokyo, Japan) to detect polymorphic bands. A SNaPshot primer extension method was performed following the manufacturer's instructions (PE Biosystems). To obtain a purified template for SNaPshot and sequencing, we removed dNTPs and primers from amplified genomic DNA by means of ExoSAP-IT (USB, Cleveland, OH, USA). In the case of the SNaPshot method, reaction mixtures were electrophoresed on an ABI 310 genetic analyzer and data were analyzed with GeneScan software (PE Biosystems). For haplotype analysis, a total of 54 DNA samples (108 alleles) of healthy volunteers, recruited in our medical school, were genotyped. All of them were medical students and gave their informed consent. The Ethics Committee of Asahikawa Medical College approved this study.

Haplotype analysis

Estimation of haplotype frequency was performed by the maximum-likelihood method using SNPAllyze (DYNACOM, Yokohama, Japan, <http://www.dynacom.co.jp/>).

Results and discussion

By sequencing the *CL-P1* gene region of ten Japanese volunteers (20 alleles), we identified five SNPs, two in intron 2 (SNP2, SNP3), one in exon 5 (SNP4), and two in exon 6 (SNP5, SNP6; Fig. 1, Table 1). In consideration of their importance in gene regulation, 20 SNPs were screened by sequencing the 5'-upstream region described in The SNP Consortium Ltd database. SNP1 was shown to be useful for our purposes. A total of six SNPs were used for genotyping and haplotype analysis. Genotype and allele frequencies of six SNPs and their location in the gene are shown in Table 1. All genotype frequencies conformed to Hardy-Weinberg equilibrium. Genotyping of 54 individuals (108 alleles) revealed that all SNPs are quite common, the minor allele frequency being at least 29%.

Of six SNPs, three (SNP4–6) are located in the coding region, one silent mutation is located in exon 5 (SNP4: T267T), and two missense mutations are located in exon 6 (SNP5: S522P, SNP6: G606S; Table 1). As shown in Fig. 1, the SNP5 in exon 6 is in the collagen-like domain that has a characteristic Gly-X-Y repetitive pattern, in which X and Y can be any amino acid but are frequently prolines or hydroxyprolines. SNP5, either serine or proline, located in the X position, could have some effect on the stability of collagen structure. In fact, in the *MBP* gene, which belongs to one of the collectin groups and is known to have a significant role in innate host immunity, three relatively common mutations in the homotrimeric region have been reported to be associated with invasive pneumococcal disease (Roy

Fig. 1. Relationship between genomic structure and the location of single-nucleotide polymorphisms (SNPs) in the human *CL-P1* gene. Vertical rectangles and horizontal lines represent exons and introns, respectively. Coding regions are shown with filled rectangles. Arrows

indicate approximate position of each SNP. Functional domains are indicated above the gene. TM, Transmembrane domain; CRD, carbohydrate recognition domain

Table 1. Genotype and allele frequencies of six SNPs in Japanese subjects

Polymorphism	Nucleotide position	Amino acid	Genotype	Allele	Allele frequency
SNP1 (5' upstream region)	-4078 ^a		TT TG GG 22 24 8	T G 68 40	T G 0.63 0.37
SNP2 (intron 2)	IVS2+814G>A ^b		GG GA AA 21 24 9	G A 66 42	G A 0.61 0.39
SNP3 (intron 2)	IVS2-1619A>G ^b		AA AG GG 24 24 6	A G 72 36	A G 0.67 0.33
SNP4 (exon 5)	800+1C>T ^b	T267T	CC CT TT 28 21 5	C T 77 31	C T 0.71 0.29
SNP5 (exon 6)	1563+1C>T ^b	S522P	CC CT TT 27 23 4	C T 77 31	C T 0.71 0.29
SNP6 (exon 6)	1815+1A>G ^b	G606S	GG GA AA 23 27 4	G A 73 35	G A 0.68 0.32

SNP, Single-nucleotide polymorphism

^aNucleotide position is identified from the translation initiation site

^bThe designation of other SNPs follows the recommendation of mutation nomenclature in Dunnen and Antonarakis (2000)

et al. 2002). Although two of the three mutations, those in codons 54 and 57, located in the Gly position, the remaining mutation in codon 52 is located at the X position, which exchange the amino acid cysteine for arginine. In terms of SNP6 in exon 6, either glycine or serine is located in the neck domain. Hydrophobic amino acids in the neck domain contribute to form an alpha-helical coiled coil. A coiled-coil structure can be recognized by its characteristic heptad repeat pattern a-b-c-d-e-f-g, in which "a" and "d" are hydrophobic residues. Each alpha-helix interacts with a neighboring CRD (Sheriff et al. 1994). Human MBP and SP-D have such heptad repeats in the neck domain (Zhang et al. 2001). However, in the case of hCL-P1, the neck domain of 17 amino acid residues does not have a characteristic heptad structure. Even so, both collagen-like domain and CRD have a collaborative role in recognizing pathogens, and an amino acid alteration in the neck domain might still have some influence.

The result of linkage disequilibrium (LD) analysis is summarized in Table 2. Among the SNPs, only SNP4 and SNP5 showed significant LD ($r^2 = 0.823$). Haplotype frequencies among 108 alleles were calculated (Table 3). The top eight haplotypes with frequencies over 5% accounted for 67% of all haplotypes.

CL-P1 is a member of the collectin family, which is considered to play significant roles in innate immunity. Classical collectins are soluble, but CL-P1 is membrane bound.

Table 2. Analysis of linkage disequilibrium for all possible two-way comparisons among six SNPs

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
SNP1	D'	0.040	0.659	0.144	0.245	0.208
	r^2	0.001	0.119	0.005	0.014	0.012
	P	0.822	0.000	0.534	0.287	0.279
SNP2	D'		0.003	0.226	0.145	0.003
	r^2		0.000	0.032	0.013	0.000
	P		0.822	0.040	0.236	0.822
SNP3	D'			0.325	0.437	0.092
	r^2			0.092	0.167	0.008
	P			0.001	0.000	0.351
SNP4	D'				0.907	1.000
	r^2				0.823	0.195
	P				0.000	0.000
SNP5	D'					1.000
	r^2					0.195
	P					0.000

CL-P1 might bind and control not only bacteria and yeasts, but also modified LDLs in the vascular space. The collagen-like domains in human and mouse CL-P1, which have the highest identity (96%) described to date, may play the most important role in these biological functions (Ohtani et al. 2001).

In conclusion, these six polymorphisms, their haplotype, and the state of LD will be useful for investigation of a possible relationship between genetic variation at the

Table 3. Frequencies of haplotypes constructed from six SNPs

SNP1 TG	SNP2 AG	SNP3 GA	SNP4 TC	SNP5 CT	SNP6 GA	Frequency
G	G	A	C	C	G	0.155
T	G	G	T	T	G	0.114
T	G	A	C	C	G	0.091
T	G	A	C	C	A	0.074
T	G	G	C	C	A	0.060
T	A	A	C	C	G	0.059
G	A	A	T	T	G	0.053
T	A	G	C	C	A	0.053
G	A	A	C	C	A	0.049
G	G	A	C	C	A	0.046
T	A	A	C	C	A	0.038
T	A	A	T	T	G	0.037
T	G	A	T	T	G	0.032
G	A	A	C	C	G	0.026
T	A	G	T	T	G	0.021
T	G	G	C	T	G	0.019
G	A	G	T	T	G	0.016
T	G	G	C	C	G	0.013
G	A	G	C	C	G	0.012
T	A	G	C	C	G	0.012
G	A	A	T	C	G	0.009
T	A	A	T	C	G	0.009
Total						1.000

human *CL-P1* locus and human diseases, especially in association studies between specific polymorphisms and susceptibility to innate immunity disease or atherosclerosis.

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