ORIGINAL ARTICLE

Association of the *CD14* gene –159C polymorphism with progression of IgA nephropathy

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The risk factors associated with the progression of IgA nephropathy (IgAN), the most common form of glomerulonephritis, are unclear. It has been suggested that CD14 signalling in response to various microbes affects the natural history of chronic inflammatory conditions. It has been hypothesised that variants in the promoter region of the *CD14* gene might alter the expression of CD14, and this in turn could influence the progressive nature of IgAN.

PCR-RFLP was used to determine the polymorphism at the –159 site (T to C). The distribution of the *CD14*/–159 polymorphism was no different in patients with IgAN (n=216) compared to 171 healthy controls. After follow up for 86 months, it was found that an excess of the C genotype occurred in patients with progressive disease (p=0.03) and the risk of disease progression increased as the number of C alleles increased (p for trend = 0.002). The hazard ratio for progression in the patients with the CC genotype was 3.2 (p=0.025) compared with the patients possessing the TT genotype. After LPS stimulation, sCD14 was released more abundantly from the PBMCs of the TT subjects than from that of the CC subjects (p=0.006), even though mCD14 expression level was no different. In addition, the TT subjects released less IL-6 than the CC subjects after stimulation (p=0.0003). These results suggest that the *CD14*/–159 polymorphism is an important marker for the progression of IgAN and may modulate the level of the inflammatory responses.

IgA nephropathy (IgAN) is the most common glomerulonephritis. It is known to be an important cause of end stage renal disease.^{1–4} Although the factors responsible for the progression of renal disease have not been fully clarified, systemic hypertension and increased urinary protein excretion indicate a poor outcome.^{1–5} It has been suggested that genetic factors may contribute not only to the susceptibility to IgAN but also to its prognosis.^{4–6} Identifying the risk factors for chronic renal disease might help to provide a therapeutic strategy. In this regard, the role of genetic polymorphisms if certain candidate genes can be found to be involved in the progression of the disease has become an intensely studied field in recent years.

LPS, which constitutes the major part of the outer membrane of gram negative bacteria, is one of the most potent stimulators of the innate immune system. As the cellular responses to LPS occur immediately after a bacterial invasion, it may serve as an early warning signal to mobilise the immune response promptly. However, exaggerated reactions to LPS can be fatal. Membrane CD14, the LPS binding receptor, the LPS binding protein (LBP), and Toll-like receptor 4 (TLR-4), the LPS signalling receptor, are the major components for cellular LPS signalling. As the LPS recognition mechanisms are extraordinarily sensitive and potentially noxious to the host, elaborate controlling mechanisms are crucial to finely tune the cellular responses. Several modalities are adopted by the host for this purpose. Serum lipoproteins, such as HDL, are known to neutralise the biological activity of LPS.^{7–8} Recently, soluble CD14 has been shown to limit dramatically the amount of LPS that remains bound to the monocytes and substantially reduce cytokine responses.⁹ A soluble form of CD14, sCD14, is abundant in serum and is apparently derived both from secretion of CD14 in a soluble form and from the enzymatic cleavage of the GPI anchored membrane bound CD14.^{10–11} In the promoter region of the *CD14* gene, a C to T transition was identified at position –159 upstream from the major transcription site (*CD14*/–159) and

this polymorphism was reported to be associated with circulating sCD14 levels.¹²

Bacterial signals have been recently suggested to play a role in promoting T helper differentiation and polarisation at the time of development of the primary immune response.¹³ In this framework, the variants in the promoter region of the *CD14* gene might alter the CD14 expression level. This in turn can regulate the proportion of Th2 to Th1 type cells responding to the environmental stimuli, thus influencing the progressive nature of IgAN. Because the onset of IgAN is associated with viral upper respiratory infection and several renal diseases are transiently aggravated after infectious episodes, the difference in CD14 signalling in response to various infectious agents might explain the differential clinical outcomes observed in patients with IgAN. The purpose of this study was to investigate whether the C allele of *CD14*/–159 was associated with the progression of IgAN. In addition, the biological significance of this polymorphism was examined.

PATIENTS AND METHODS

Patients and controls

The study patients were recruited from the Department of Internal Medicine, Seoul National University Hospital. Two hundred and sixteen patients with primary IgAN, who had a minimum follow up period of two years and who provided informed consent, were enrolled in this study. A diagnosis of IgAN was based on the mesangial proliferation and the presence of typical immunofluorescent changes on a renal biopsy. Patients with evidence of systemic diseases such as diabetes, chronic liver disease, and systemic lupus erythematosus were excluded. One hundred and seventy-one healthy, randomly selected subjects who had no evidence of renal disease and were normotensive were also recruited from the Health Promotion Centre of the same hospital as controls. The Internal Review Board of the institution approved the research protocol used for this study. Hypertension was defined as a

Table 1 Baseline clinical characteristics of patients according to outcomes

	Stable disease (n=162)	Progressive disease (n=54)	p value*
Age (years)†	33.4±14.3	34.9±13.5	NS
Sex (male:female)	85:77	37:17	NS
Follow up duration (months)	86.2±51.7	86.6±49.9	NS
Hypertension†‡	35.1%	64.0%	0.0003
Therapy with anti-RAS§	43.4%	39.2%	NS
Initial serum creatinine (mg/dl)†	1.07±0.39	1.55±0.67	<0.0001
Daily proteinuria (mg/day)†	1880±2184	2970±2606	0.008

*Mann-Whitney test for continuous variables, χ^2 test for discrete variables. †At the time of histological diagnosis. ‡Systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or needing antihypertensive drugs to maintain normal blood pressure. §Therapy with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers.

systolic blood pressure of >140 mmHg or a diastolic pressure of >90 mmHg, or if antihypertensives were necessary to maintain normal blood pressure. Baseline clinical parameters reported in this work were those evaluated at the time of histological diagnosis. The progression of renal disease was defined when the serum creatinine level had increased to twice the basal serum creatinine level during the follow up or there was a requirement for renal replacement therapy.

Extraction of genomic DNA and genotype determination

DNA was extracted using standard protocols (Wizard Genomic DNA purification kit, Promega, Madison, WI, USA). Genotyping of the *CD14*-159 polymorphism was performed according to the protocol described by Koppelman *et al.*¹⁴ Briefly, a polymerase chain reaction was performed in 30 µl volumes containing 50 ng of DNA, 250 µmol/l dNTP, 1.5 mmol/l MgCl₂, 10 × buffer, 0.5 U *Taq* polymerase (Takara Shuzo Co, Kyoto, Japan), and 0.1 µmol/l sense primer 5'-GCCTCTGACAGTTTATGTAATC-3' and primer 5'-GTGCCAA CAGATGAGGTTAGTTTATGTAATC-3'. The cycling conditions used were 94°C for five minutes, 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for five minutes. The PCR amplified DNA was digested with 5 U *Ava*II and 1 µl of the manufacturer's buffer (New England Biolabs Inc, Beverly, MA) at 37°C for two hours. The products were separated on a 1.5% agarose gel. The DNA was visualised using a single intensity transilluminator (300 nm) and photographed with a Gel-Doc system (Bio-Rad Laboratories, Hercules, CA, USA). *Ava*II digests the PCR product only when the T allele is present. The uncut product is 497 bp whereas the digested products are 144 and 353 bp. The results of this restriction fragment length polymorphism assay were confirmed by direct sequencing of the -159 promoter region of the *CD14* gene in 10% of patients and controls (Macrogen Inc, Seoul, Korea).

Cell stimulation and ELISA assays

The mCD14, sCD14, and IL-6 levels were measured after LPS stimulation in order to assess the cellular responses against external stimuli. Blood was drawn from the normal subjects whose *CD14*-159 polymorphism was determined by the PCR-RFLP method. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (Sigma, MO, USA) and stimulated with LPS (Sigma, 50 mg/ml). After 24 and 48 hours, the PBMCs were harvested and the culture supernatants were collected. Anti-CD14 antibody (BD, NJ, USA) was used for flow cytometry. sCD14 and IL-6 were measured using a commercially available, enzyme linked immunosorbent assay (ELISA) kit (Quantikine™, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All data were analysed using SAS version 6.12 (SAS Institute Inc, Cary, NC, USA) and are presented as mean (SD). The

groups were compared using Student's *t* test or a non-parametric test, such as the Kruskal-Wallis and Mann-Whitney tests for continuous variables or the χ^2 test for discrete variables. The Mantel-Haenszel χ^2 test was used to detect the differences in the genotype distributions of the patients and controls. A log rank test and the Cox proportional hazards models were used to compare the risks of developing the progressive disease for the three groups of genotypes. A p value <0.05 was considered statistically significant. Cox's proportional hazard model was performed to determine the effect of each variable on the renal outcome. The hazard ratios were used to estimate the risk of an association between the renal outcome and each variable.

RESULTS

Sixty-four out of 216 patients (30%) with IgAN were homozygous for thymine (TT) at position -159 (-159TT). One hundred and ten patients (51%) were heterozygous (-159TC) and 42 (19%) were homozygous for cytosine (-159CC). These genetic frequencies did not differ significantly from those of normal controls: 51 (29.8%) out of 171 controls for -159TT, 82 (48.0%) for -159TC, and 38 (22.2%) for -159CC (p=0.92, Mantel-Haenszel test). Hardy-Weinberg equilibrium was attained for these analyses (p=0.81).

The average (SD) period of follow up was 86 (SD 51.1) months. The patients were segregated into two subgroups on the basis of a progression of underlying disease. Fifty-four patients (25%) had progressive renal disease (PD) and the other 162 patients (75%) had stable renal disease (SD). The age of the patients at the time of biopsy was not statistically different between the two groups (p=0.59). Patients with PD were shown to have a significantly higher baseline serum creatinine level (1.55 (SD 0.67) v 1.07 (SD 0.39) mg/dl, p<0.001) and more daily proteinuria (2970 (SD 2606) v 1880 (SD 2184) mg, p=0.008) than patients with SD. In addition, the presence of hypertension was more prevalent in the PD group than in the SD group (59% v 33%, p<0.001, table 1).

To determine whether or not the risk for developing the progressive disease increased as the number of C alleles increased, survival analysis methods were used. Kaplan-Meier survival curves were estimated using the number of dates reaching the primary end point (doubling the baseline serum creatinine) according to the *CD14* genotype (fig 1). During the follow up period, patients with the CC genotype of *CD14* lost their renal function more frequently as determined by the end point versus those who had the TC or TT genotype of *CD14* (p=0.03, log rank test). The Cox proportional hazards model confirmed that risk for developing the progressive disease increased linearly as the number of C alleles increased (p=0.0023 for linear trend). The mean duration of follow up was similar for the PD and SD patients, 86 (SD 49.9) and 86 (SD 51.7) months, respectively (table 1). *CD14* genotypes were examined to determine whether or not there was any differences between the two subgroups. Fifteen out of 54

Figure 1 Probability of patients with IgAN not reaching doubling of baseline serum creatinine according to the genotypes of *CD14* (Kaplan-Meier analysis, $p=0.03$ by log rank test).

Table 2 Association of genetic polymorphisms of *CD14* and progression of IgA nephropathy

	Stable disease (n=162)	Progressive disease (n=54)
Genotypes of <i>CD14</i> *		
CC	27 (16.7%)	15 (27.8%)
TC	80 (49.4%)	30 (55.6%)
TT	55 (34.0%)	9 (16.7%)

* $p=0.03$ by χ^2 test.

patients (28%) with PD had the -159CC genotype, 30 out of 54 (56%) were TC, and nine patients (16%) had the -159TT genotype, whereas, 55 out of 162 patients (34%) with stable disease had the -159TT genotype (table 2). *CD14* genotypes were not associated with any clinical characteristics in patients with IgAN (table 3). Univariate regression analysis showed that the presence of hypertension, the amount of daily proteinuria, renal function at the time of renal biopsy, and the *CD14* genotypes were significantly associated with the progression of renal disease. Other clinical parameters, such as age and gender, were proved insignificant by univariate analysis (data not shown). Cox proportional hazard analysis identified the *CD14* genotypes, the amount of daily proteinuria, and the renal function at the time of renal biopsy as being significant predictive variables for the progression of the disease. The hazard ratio for the progression of renal disease in patients with the CC genotypes was 3.2 ($p=0.025$) and 1.7 ($p=0.21$) in patients with the TC genotype compared with patients with the TT genotype (table 4). Furthermore, the presence of massive proteinuria (>3000 mg/day) affected the progression of the disease (hazard ratio 4.0).

Table 4 Predictors for the progression of renal disease by the Cox proportional hazards analysis

Variables	Hazard ratio	p value	95% confidence interval
Proteinuria*†			
>3000 mg/day	4.0	0.0004	1.8 to 8.5
1000–3000 mg/day	1.6	0.28	0.7 to 3.9
Genotype of <i>CD14</i> ‡			
CC	3.2	0.025	1.2 to 8.8
TC	1.7	0.21	0.7 to 4.1
Creatinine*§			
>1.4 mg/dl	3.6	0.0015	1.9 to 5.8

*At the time of histological diagnosis. †Compared with proteinuria <1000 mg/day. ‡Compared with TT genotype. §Compared with ≤ 1.4 mg/dl.

To assess the functional significance of the *CD14*-159 polymorphisms, the membrane bound form of *CD14* (m*CD14*) and soluble *CD14* (s*CD14*) from PBMCs were measured using flow cytometry and ELISA, respectively. Baseline m*CD14* and s*CD14* levels before stimulation were comparable among the different genotypes (data not shown). Even after LPS stimulation (50 ng/ml), the m*CD14* fluorescent intensity was not different in terms of the different genotypes of *CD14*-159 (fig 2A). However, s*CD14* was released more abundantly from the PBMCs of the TT subjects than from the CC subjects (8620 (SD 2632) v 5390 (SD 2059) pg/ml, $p=0.006$) (fig 2B).

As s*CD14* can diminish cell responses to LPS, the different s*CD14* levels were correlated with LPS responsiveness among the different genotypes. The IL-6 levels from the culture supernatants of the PBMCs stimulated with LPS (50 ng/ml) were measured. After 24 hours of stimulation, the PBMCs from the CC subjects released more IL-6 than from the TT subjects (5550 (SD 397) v 3080 (SD 721) pg/ml, $p=0.0003$) (fig 3). These different responses according to the *CD14* genotypes were maintained after 48 hours of stimulation (data not shown).

DISCUSSION

This study confirms the association of the *CD14*-159 promoter polymorphism with the progression of IgAN. Differential expression of IL-6 as well as s*CD14* was also found according to the genotypes of the *CD14* promoter region.

A new aspect of research in human genetics involves associating the sequence variations with specific human diseases. Single nucleotide polymorphisms (SNPs) are probably the most common of these variations.¹⁵ Genome wide association studies for the SNPs have been made in an effort to identify the complex disease and/or pharmacogenomic applications. In this regard, it is proposed that the polymorphic nucleotide in the -159 site of *CD14* be identified as a marker for the progression of IgAN. Although many genetic factors have been

Table 3 Clinical characteristics of patients according to the *CD14* genotypes

	<i>CD14</i> genotypes			
	CC	TC	TT	p value*
Age (years)†	33.0±15.8	34.4±13.7	33.1±13.8	NS
Sex (male:female)	24:18	65:45	33:31	NS
Follow up duration (months)	78.9±56.3	88.5±50.1	87.2±49.5	NS
Hypertension†‡	50.0%	39.8%	41.3%	NS
Therapy with anti-RAS§	38.9%	40.4%	47.5%	NS
Initial serum creatinine (mg/dl)†	1.34±0.63	1.21±0.56	1.04±0.29	NS
Daily proteinuria (mg/day)†	1799±1807	2148±2246	2388±2775	NS

*Kruskal-Wallis test for continuous variables, χ^2 test for discrete variables. †At the time of histological diagnosis. ‡Systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or needing antihypertensive drugs to maintain normal blood pressure. §Therapy with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers.

Figure 2 Differential expressions of membrane bound CD14 (mCD14) (A) and soluble CD14 (sCD14) (B) according to the genotypes of *CD14* after LPS stimulation. PBMCs harvested from normal controls were stimulated with LPS (50 ng/ml) for 24 hours. PBMCs from CC genotypes released less soluble CD14 than from TT genotypes (mean (SD)). Each group contains four to six subjects. This is representative of the results obtained in three separate experiments. * $p=0.006$ by Kruskal-Wallis test.

Figure 3 Levels of IL-6 according to the genotypes of *CD14* with LPS stimulation. Human PBMCs from normal controls were stimulated with LPS (50 ng/ml) for 24 hours. Secretion of IL-6 from CC genotypes was more abundant than from TT genotypes (mean (SD)). Each group contains four to six subjects. This is representative of the results obtained in three separate experiments. * $p=0.003$ by Kruskal-Wallis test.

suggested to be associated with a poor prognosis of IgAN,^{16–18} limitations inherent in the reported studies, such as the sample size, the follow up period, a variable end point, and technical problems, made it difficult to draw clear conclusions. Patients with –159CC and –159TC polymorphisms were found to have an increased risk of IgAN progression. Moreover, homozygosity for the CC polymorphism appeared to have a

profound harmful effect on the progression of renal disease. These associations are independent of age, gender, and other clinical manifestations, such as the daily proteinuria or the presence of hypertension. We used the creatinine doubling as a surrogate marker for renal disease progression, although the slope of creatinine over time is a more accurate marker. It would be a potential flaw. We tried to resolve this problem through a relatively large patient sample size and long follow up. The clinical presentation was no different in the patient groups according to *CD14* genotypes and the frequencies of each genotype between the patients and normal controls were similar. Thus, it is proposed that the SNP of *CD14* does not influence the development of IgAN, but does affect disease progression, which would fit the biological properties of sCD14. Because of an inherent inability to discriminate between the self versus non-self, the adaptive immune system requires previous conditioning through the innate immune system. *CD14* has thus been revisited as a pattern recognition receptor that could directly recognise foreign microbes.¹⁹ Through complex interactions with LBP, TLR4, and MD-2,²⁰ *CD14* plays a central role in mediating the cellular response to LPS, the outer membrane component of gram negative bacteria. As several bacterial and viral components are also suggested to exert their activities through *CD14*,^{21–23} it stands in the first line of the immune response. Macrophages/monocytes, with their surface *CD14* molecules, are the key cellular components that initiate the inflammatory response to microbial products. They then produce numerous cytokines and growth factors.²⁴ With an infection, as with other risk factors for the progression of renal disease, the genetic makeup of each person should influence the sensitivity to this stimulus.

IgAN is characterised by mesangial IgA deposition. The exact nature of the antigenic specificity of pathogenic IgA is still unknown, but they are polyclonal and are mostly composed of the IgA1 form. Even though many reports suggest its association with concurrent microbial infections,²⁵ the causative links between them are still unknown. A comparable ratio of the different CD14/–159 genotypes was found between the patients and controls in this study, so differences in the CD14 level play a minor role in the initiation process of IgAN. However, an excess of the C genotype that was found in the progressive group in this study might imply possible links between the sCD14 levels and the disease severity. As the key pathological profile of IgA nephropathy is the mesangial cell response to deposited immunoglobulin, modulating the mesangial responsiveness through sCD14 might affect the disease progress in different genotypes. We found that the sCD14 levels in the sera from patients were not different according to CD14/–159 genotypes in the unstimulated state (data not shown), but they were differentially regulated after stimulation in normal subjects (fig 2B). Also the differential production of IL-6 from the mononuclear cells after LPS stimulation further supported the possible link between the biological properties of sCD14 and the magnitude of responses by host. It is believed that after the initial pathogenic immunoglobulin deposition in the glomeruli, the mesangial responsiveness to a common systemic infection might affect the progress of IgAN, and the sCD14 levels dictated by the amount of C at the CD14/–159 position would affect the severity and progression of the kidney pathology. CD14/–159 is near a SP1 binding site that has a major influence on monocyte specific expression of CD14.²⁶ There are several plausible explanations for the possible role of CD14 in glomerulonephritis. One of the possibilities is that because CD14 on the surface of monocytes and polymorphonuclear cells functions as receptors for LPS, and because these cells produce IL-6 and IL-8, CD14 may be involved in a proinflammatory pathway through the release of cytokines.²⁷ It has been reported that the polymorphism of CD14/–159 was associated with the expression of a more severe allergic phenotype,¹⁴ the development of ulcerative colitis,²⁸ and susceptibility to septic shock.²⁹

In conclusion, this study identified a significant relationship between the genotype differences at position –159 of the CD14 gene and the risk of IgAN progression in the Korean population. The polymorphism may alter sCD14 expression and influence the inflammatory responses.

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