Association of interleukin-10 gene G-1082A polymorphism with the progression of primary glomerulonephritis

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Background. Interleukin-10 (IL-10) is a cytokine with immunosuppressive properties. We evaluated the influence of G-1082A polymorphism in the IL-10 gene promoter, which has been associated with modified IL-10 production, on the two most common forms of primary glomerulonephritis: IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS).

Methods. We studied Caucasian patients (N = 191) with biopsy-proven glomerulonephritis (IgAN: N = 123, FSGS: N = 68) followed-up for 6.5 ± 5.5 years. Patients were classified according to the slope of reciprocal serum creatinine (≥ or < 0.1 dL-mg⁻¹-year⁻¹) into group A (slow progressors, IgAN: N = 75, FSGS: N = 47) and group B (fast progressors, IgAN: N = 48, FSGS: N = 21). One hundred healthy volunteers were analyzed as control patients. G-1082A polymorphism was determined by polymerase chain reaction (PCR) amplification.

Results. The allele frequencies were similar in patients and control group (NS). Initial renal function, proteinuria, and blood pressure did not differ significantly between patients with different genotypes. G-1082A polymorphism was associated with the progression of both IgAN and FSGS: GA/AA genotypes were more frequent in group B (fast progressors) than in group A (slow progressors; P = 0.012 for IgAN, P < 0.05 for FSGS). Patients with the GA/AA genotypes showed a worse outcome in the Kaplan-Meier analysis of renal survival (P < 0.05 for both IgAN and FSGS). The IL-10 polymorphism remained an independent risk factor for progression in multivariate analysis (Cox regression model, P < 0.05 for IgAN and FSGS).

Conclusion. Our results suggest that IL-10 gene G-1082A polymorphism is an important marker of progression in patients with IgAN and FSGS.

Primary glomerulonephritis remains one of the major causes of end-stage renal disease (ESRD). The two most common forms of primary glomerulonephritis are IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS) [1, 2]. IgAN is characterized by mesangial deposition of immunoglobin A, matrix expansion, and cellular proliferation. The clinical course of IgA nephropathy is variable, ranging from stable renal function over decades to terminal renal failure within a few years. About 10% to 20% of the patients progress to end-stage renal disease (ESRD) in the first decade after renal biopsy [1]. Clinical predictors including impairment of renal function, severe proteinuria, and arterial hypertension at presentation have been associated with an unfavorable outcome [3]. Primary focal segmental glomerulosclerosis refers to a specific renal histologic lesion [i.e., sclerosis found in a portion (segment) of some (focal) glomeruli without identifiable underlying cause]. It appears to be a disease with different entities, which are difficult to distinguish in morphologic terms. Whereas two thirds progress to ESRD within five to 10 years, others have a more benign course with complete remission in a short time, or slow progression over many years. Efforts to identify these two groups have led to the identification of risk factors such as increased plasma creatinine on diagnosis, high proteinuria, and therapy-refractory nephrotic syndrome, but clear-cut criteria for an individual prognosis and targeted therapy have not yet been established [2]. The pathogenetic mechanisms of both IgAN and FSGS are still under research. Several factors point to the possible role of genetic influences on their development and outcome. Familiar forms of both IgAN [4] and FSGS [5] have been described. A linkage of IgAN to chromosome 6q22-q23 has been demonstrated [6], and the genetic disorder underlying the “Finnish type” of hereditary FSGS [7] and autosomal-recessive steroid-resistant nephrotic syndrome [8] has been identified. Furthermore, there is a growing body of evidence supporting a pivotal role of cytokines and chemokines in glomerular inflammation in primary glomerulonephritis [9].

Interleukin 10 (IL-10) is a pleiotropic cytokine produced by T-helper type 2 (Th2) cells, B cells, monocytes, and macrophages. It inhibits a broad array of immune

Key words: IgA nephropathy, focal segmental glomerulosclerosis, interleukin-10, genetic polymorphism, G-1082A polymorphism.

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parameters including Th1 lymphocyte cytokine production, antigen presentation, and antigen-specific T-cell proliferation, and could play a role as a natural blocking agent in the inflammation mediated by proinflammatory cytokines [10]. The mechanisms regulating IL-10 production are still under study. It appears that different cells respond differently to each stimulus, and their response depends on the cell type and the microenvironment of the cell. Like many other cytokines, the amount of IL-10 produced on a specific stimulus is subject to genetic regulation. Turner et al demonstrated a difference in IL-10 secretion in vitro in association with the presence or absence of the A-allele at position −1082 of the human IL-10 promoter after concanavalin A stimulation of peripheral blood mononuclear cells [11]. Reduced IL-10 serum levels were also observed in vivo in the “low IL-10 producer” GA/AA genotypes (presence of −1082A allele) [12].

Because of its regulatory functions in both cellular and humoral immune responses, IL-10 is a candidate mediator in controlling renal inflammation. In the present study, we evaluated the role of the IL-10 gene G-1082A polymorphism in the clinical course of IgAN and FSGS. Our aim was to identify genetically determined subgroups of patients with primary glomerulonephritis who had a more rapid decline in renal function and earlier onset of ESRD. The identification of genetic factors that may explain the clinical variability of IgAN and FSGS is critical for understanding their pathogenesis and formulating future therapeutic strategies.

METHODS
Patients and control patients

We studied Caucasian patients (N = 191; 128 males and 63 females) with biopsy-proven primary glomerulonephritis (IgA: N = 123, FSGS: N = 68) treated in our center from 1968 to 2003. The mean follow-up period was 6.5 ± 5.5 years, and included monitoring of serum creatinine, endogenous creatinine clearance (24-hour urine collection), proteinuria, blood pressure, and antihypertensive medication. Arterial hypertension was defined by the presence of blood pressure values over 140 mm Hg systolic and/or 90 mm Hg diastolic or the need for antihypertensive medication. Arterial hypertension was defined by the presence of blood pressure values over 140 mm Hg systolic and/or 90 mm Hg diastolic or the need for antihypertensive treatment. A mean of 19.5 ± 6 years, and included monitoring of serum creatinine [13]. A Cox regression model was calculated as the slope of reciprocal serum creatinine versus time plot (linear regression). The Kaplan-Meier method using a log-rank test was employed for survival analysis of the kidney as a functioning organ. The end points included ESRD or doubling of the initial serum creatinine [13]. A Cox regression model was used for multivariate analysis. The following covariates were tested: sex, age, serum creatinine, proteinuria, and blood pressure at the time of renal biopsy, and IL-10 gene polymorphism. Statistical analysis was performed using

Determination of IL-10 gene G-1082A polymorphism

Genomic DNA was extracted from peripheral leukocytes from whole blood samples using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Two different polymerase chain reaction (PCR) amplifications, each one specific for each allele, were carried out using the following primers: generic primer (antisense): 5′ CAGTGCA AACTGAGAATTGG 3′; sense primer: G-allele specific PCR: 5′ CTACTAA GGGCTCTTTGGA 3′, A-allele specific PCR 5′ ACTACTAAGGCTTTGGA AA 3′ (MWG Biotech AG, Ebersberg, Germany). Thermocycling included an initial denaturation step (5 minutes at 95°C) and 30 cycles consisting of 30 seconds at 95°C, 40 seconds at 65°C, and 70 seconds at 72°C, followed by a final extension step at 72°C for 7 minutes. A 661-bp long sequence of the β-actin gene was amplified as an internal control in each sample. The combined agarose gel electrophoresis of the PCR products (both 258-bp long) allowed determination of the G-1082A genotype. Internal and external controls were used in order to prohibit misclassifications during genotyping. All PCR protocols were evaluated by two independent investigators who were blinded for the phenotype of the samples.

Statistical analysis

Data were expressed as percentages or mean ± standard deviation. Pearson’s chi-square was used for categorical data. Continuous variables were tested in each group for normal distribution using the Kolmogorov-Smirnov test for one variable. Differences between two groups were tested with the Student t test or the nonparametric Mann-Whitney U test. The expected allele frequencies under the assumption of the Hardy-Weinberg equilibrium were compared with the observed frequencies in each study population. Both genotype and allele frequencies were compared in the different groups in order to reduce the possibility of spurious associations. The individual rate of progression of renal insufficiency was calculated as the slope of reciprocal serum creatinine versus time plot (linear regression). The Kaplan-Meier method using a log-rank test was employed for survival analysis of the kidney as a functioning organ. The end points included ESRD or doubling of the initial serum creatinine [13]. A Cox regression model was used for multivariate analysis. The following covariates were tested: sex, age, serum creatinine, proteinuria, and blood pressure at the time of renal biopsy, and IL-10 gene polymorphism. Statistical analysis was performed using
involved 68 Caucasian patients, 33 women and 35 men, with biopsies confirmed as primary FSGS. Their mean age at diagnosis was 41.2 ± 18 years. The mean serum creatinine was 1.5 ± 0.9 mg/dL, the creatinine clearance 90.2 ± 35 mL/min/1.73 m², and the mean proteinuria 11.2 ± 10.2 g/24h. Forty-seven patients with FSGS belonged to group A (slow progressors), and N = 21 to group B (fast progressors). Regarding treatment, 62.2% of the patients in group A received ACE inhibitors compared with 60.0% in group B (NS). Furthermore, there were no relevant differences concerning the number of patients treated with immunosuppressive drugs (corticosteroids: group A: 21.3%, group B: 14.3%; corticosteroids and cyclosporine: group A: 53.2%, group B: 66.7%, NS). At the time of genotyping, N = 27 patients (40%) had reached ESRD, and N = 10 (15%) had reached only the second end point (doubling of the initial serum creatinine). Increased serum creatinine (Cox regression: P < 0.001) and higher blood pressure values (P < 0.01) were associated with a worse prognosis in our patients with FSGS. As in the case of IgAN, patients with FSGS diagnosed in the last 15 years tended to have more rapidly declining renal function.

### IL-10 genotyping and parameters at the time of renal biopsy

The allele frequencies of G-1082A polymorphism were similar in control subjects (G-allele: 0.43, A-allele: 0.57) and patients with IgAN (G-allele: 0.41, A-allele: 0.59, NS) or FSGS (G-allele: 0.52, A-allele: 0.48, NS). In all groups the observed genotype frequencies corresponded to the expected values according to the Hardy-Weinberg equilibrium. There were no relevant differences at the time of renal biopsy in terms of renal function, proteinuria, and blood pressure between patients with different G-1082A genotypes in both IgAN and FSGS (Table 1, NS). Patients with FSGS and the GA/AA genotypes underwent renal biopsy at a younger age (P < 0.05,
Table 1). No significant difference in the number of patients with different G-1082A genotypes treated with ACE inhibitors or immunosuppressive agents was observed in either glomerulonephritis forms (Table 1, NS).

**IL-10 genotype and progression**

*IgA nephropathy.* G-1082A polymorphism was associated with the progression of IgAN, with the GA/AA genotypes (carriage of the A-allele) being associated with a worse prognosis. The GA/AA genotypes were significantly over-represented in group B (fast progressors, OR for the GA/AA genotypes: 1.25, 95% CI 1.07–1.47, P = 0.012) (Fig. 1). The frequency of the A-allele was also significantly higher among fast progressors (G-allele: 0.30, A-allele: 0.70) than in slow progressors (G-allele: 0.47, A-allele: 0.53, P < 0.01). In the Kaplan-Meier analysis of kidney survival, patients with the GA/AA genotypes showed a significantly worse outcome (6.3 ± 0.8 years) compared with the GG genotype (11.4 ± 2.8 years, mean ± SE, P < 0.05) (Fig. 2). The IL-10 polymorphism remained an independent risk factor for progression in multivariate analysis (Cox regression model), including initial serum creatinine, proteinuria, and blood pressure (HR for low IL-10 GA/AA genotypes: 1.48, 95.0% CI 1.02–1.81, P < 0.05).

**Focal segmental glomerulosclerosis.** A significant association of IL-10 gene polymorphism with the progression of FSGS was also observed. The GA/AA genotypes was significantly more frequent among patients with FSGS belonging to group B (fast progressors) compared to group A (slow progressors, OR for the GA/AA genotypes: 1.44, 95% CI: 1.07–1.93, P < 0.05, figure 3). The frequency of the A-allele was also significantly higher in group B (0.62) than in group A (0.43, P < 0.05). A worse outcome was observed in the Kaplan-Meier analysis of renal survival in patients carrying the GA/AA genotypes (6.2 ± 0.8 years) than in the GG genotype genotype (10.6 ± 1.6 years, mean ± SE, P < 0.05) (Fig. 4). In a Cox regression model IL-10 polymorphism remained as an independent risk factor for progression (multivariate analysis including initial serum creatinine and blood pressure; HR for the GA/AA genotypes: 1.57, 95.0% CI 1.03–2.41, P < 0.05).
DISCUSSION

In view of the important role that cytokines are thought to play in the development of renal injury, we assessed the effects of a genetic variant of IL-10 gene on the progression of IgA nephropathy and focal segmental glomerulosclerosis. We found evidence of genetically determined subgroups of patients predisposed to accelerated loss of renal function and earlier onset of ESRD.

IgA nephropathy is initiated by glomerular deposition of polymeric IgA. In view of the wide clinical range of IgAN, it may be assumed that there is more than one pathogenic mechanism, and that mesangial IgA deposition may well be a final common pathway for more than one type of IgA immune system abnormality [14]. Altered metabolism of polymeric IgA (decreased production by the mucosa [15] and increased by bone marrow [16]), and impaired IgA glycosylation and clearance [17] have been implicated. Although the events that lead to IgA deposition and the initiation of glomerular inflammation are thought to be specific to IgAN, subsequent processes involving inflammatory injury and promoting renal scarring are likely to be generic (with few differences) to other chronic glomerular diseases [14]. Transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) are mainly involved in this process [18], but other cytokines such as IL-1 [19], IL-6 [19, 20], and tumor necrosis factor-alpha (TNF-α) [19] have also been implicated. Although the role of immunosuppressive agents in the therapy of IgAN is still controversial [21, 22], the reduction of proteinuria achieved in IgAN patients with normal cholesterol levels by CoA reductase inhibitors [23] underlines the significance of inflammatory mechanisms in IgAN. Likewise, although the pathogenesis of FSGS remains obscure, increasing amounts of information are becoming available regarding factors that may account for the progression of the disease. Prominent among them once again are TGF-β [24, 25] and PDGF [26], but other cytokines like TNF-α [27] seem to play an important role as well. It is therefore logical to consider polymorphisms within genes controlling this cytokine network as candidates for elucidating the variable clinical course of primary glomerulonephritis and IgAN, or FSGS in particular.

IL-10 exerts its actions on a variety of cells. Its effects are both complex and varied. The earliest described role of IL-10 was that of a “cytokine synthesis inhibitory factor” (CSIF). IL-10 was characterized as a product of Th2 cells that down-regulated the production of INF-γ, IL-2, and TNF-β by Th1 cells [28]. It decreases the surface expression of major histocompatibility complex (MHC) class II molecules on a variety of antigen presenting cells, down-regulates costimulatory pathways as intracellular adhesion molecule-1 (ICAM-1) expression, and inhibits IL-2 production by the T cells, all this resulting in decreased antigen-simulated T-cell proliferation of CD4+ Th cells. Furthermore, IL-10 inhibits the synthesis of proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-8). However, in addition to these anti-inflammatory effects, IL-10 promotes B-cell activation, regulates immunoglobulin class switching, and maintains B-cell viability by inhibiting apoptosis [10].

Higher levels [29] and higher transcriptional expression [30] of IL-10 have been demonstrated in white blood cells from patients with IgAN. Both IL-10 mRNA and IL-10 protein levels correlated to the severity of glomerular lesions in biopsies of patients with IgAN [31]. The presence of IL-10 in severe lesions may reflect activation of protective mechanisms in the context of severe glomerular injury. Knockout IL-10–deficient mice developed functionally and histologically more exaggerated glomerulonephritis in an anti-GBM (glomerular basement membrane) model [32]. In an experimental model of mesangial proliferative glomerulonephritis, administration of IL-10 diminished inflammatory cell recruitment and mesangial cell proliferation [33]. Gene transfer of the human IL-10 gene, leading to increased IL-10 expression, prevented the development of an animal model of FSGS [34]. It is not clear to what extent the anti-inflammatory and antiproliferative effects of IL-10 on renal cells are direct, and to what extent they are mediated through modulation of other cytokines, for example, through the described inhibition of TNF-α secretion from peripheral blood monocytes in patients with IgAN [35].

IL-10 gene coding for the 160 amino acid long protein has been mapped to chromosome 1 [36]. Regulatory sequences have been identified between position –1100 and –900 in the promoter of the IL-10 gene [37]. A polymorphism in the form of a single base pair substitution (substitution of guanine with adenine in codon –1082) in this promoter region was found to correlate with significant decreases in mitogen (concanavalin A) stimulated IL-10 production by peripheral mononuclear cells [11]. This mutation might alter a specific transcription factor recognition site, and consequently affect transcriptional activation and IL-10 production. The polymorphism lies within a putative external transcribed spacer (ETS) recognition site [37], and may therefore affect the binding of this transcription factor, which has been shown to act as a negative regulator of IL-2 production [38].

Increased frequency of the GA/AA genotypes was reported in patients with rheumatoid arthritis [39], Wegener’s granulomatosis [40], Crohn’s disease, and ulcerative colitis [12]. G-1082A polymorphism has also been associated with the acute rejection rate [41] and long-term graft survival [42] after renal transplantation, and the GA/AA genotypes predicted a higher cardiovascular morbidity in dialysis patients [43].

In the present study we investigated the relationship of IL-10 G-1082A genotype with clinical outcomes in patients with IgAN and FSGS. No significant difference
was observed in the allele frequencies of patients and control subjects. Thus, the examined polymorphism gave no indication of a predisposition for the development of these glomerulonephritis forms. Furthermore, there were no significant differences in initial renal function, proteinuria, and blood pressure in patients with different genotypes. Patients with FSGS carrying the GA/AA genotype, which has been associated with reduced IL-10 production in vitro [11] and in vivo [12], underwent renal biopsy at a younger age. G-1082A polymorphism was associated with the progression of both IgAN and FSGS, with the GA/AA genotypes being connected with a worse prognosis. The GA/AA genotypes were significantly over-represented among fast progressors, and patients carrying it showed a significantly worse outcome in the Kaplan-Meier analysis of kidney survival. The IL-10 genotypes remained as an independent risk factor for progression in multivariate analysis. To our knowledge, there are no earlier reports of IL-10 gene G-1082A polymorphism on the response to immunosuppressive agents. The Collaborative Study Group.

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**CONCLUSION**

This study suggests that the IL-10 gene GA/AA genotype is a risk factor for accelerated progression in Caucasian patients with IgA nephropathy and focal segmental glomerulosclerosis. IL-10 gene G-1082A polymorphism is one of the parameters that should be determined in upcoming prospective studies, and could be used in the future for a more accurate prediction of progression, and could provide new options for the clinical management of patients with primary glomerulonephritis.


