Original Article

The Influence of Vascular Endothelial Growth Factor (VEGF) Polymorphism on the Progression of Chronic Glomerulonephritides

(vascular endothelial growth factor / IgA nephropathy / focal segmental glomerulosclerosis / membranous glomerulonephritis / gene promoter polymorphism)

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Abstract. Vascular endothelial growth factor is an important mediator in maintaining normal kidney functions. In addition, several lines of evidence show that up-regulation of this mediator in glomeruli may be associated with or may directly cause renal dysfunction. We tried to assess the influence of the -2578 C/A and -1154 G/A polymorphisms in the regulatory region of the vascular endothelial growth factor gene upon progression of three primary chronic glomerulonephritides (minimal change disease/focal and segmental glomerulosclerosis, membranous nephropathy, immunoglobulin A nephropathy). We studied a cohort of 213 patients compared to 311 unrelated healthy controls. Analysis of the C/A polymorphism of vascular endothelial growth factor revealed an increased prevalence of CC genotype in the minimal change disease/focal and segmental glomerulosclerosis group in comparison with the other groups. A balanced distribution of G and A alleles among the respective types of chronic glomerulonephritides was shown in the analysis of -1154 G/A polymorphism. Finally, we have not proved any significant influence of the polymorphisms at positions -2578 C/A and -1154 G/A of the vascular endothelial growth factor gene promoter on the progression of chronic glomerulonephritides even though our study suggests a negative effect of CC genotype of -2578 C/A polymorphism on the clinical course of minimal change disease/focal segmental glomerulosclerosis.

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Abbreviations: ADPKD – autosomal-dominant polycystic kidney disease, ESRF – end-stage renal failure, FSGS – focal and segmental glomerulosclerosis, GFR – glomerular filtration rate, GN – glomerulonephritis, IgAN – immunoglobulin A nephropathy, MCD – minimal change disease, MDRD formula – modification of diet in renal disease formula, MGN – membranous nephropathy, PCR – polymerase chain reaction, S-albumin/creatinine – serum albumin/creatinine, UTR – untranslated region, VEGF – vascular endothelial growth factor, VEGFR1/2 – vascular endothelial growth factor receptor 1/2.

Introduction

Chronic glomerulonephritides (GN) form an important group of kidney diseases, in which the dominant glomerular lesion is characterized by the participation of various immunopathological mechanisms and various proportions of inflammatory and non-inflammatory processes. The spectrum of primary chronic GN ranges from nosological entities such as minimal change disease (MCD), focal and segmental glomerulosclerosis (FSGS) and membranous nephropathy (MGN), histologically characterized by a low degree of inflammatory and proliferative changes and clinically by proteinuria attaining nephrotic range on the one hand, to immunoglobulin A nephropathy (IgAN) and other GN characterized histologically by notable inflammatory and proliferative changes and clinically by nephritic syndrome on the other hand. In the pathogenesis of GN a complex interplay between immunological and non-immunological factors, histological changes and clinical signs is observed, not only in the initial process of the development of GN but also during the stepwise decrease of glomeru-

lar filtration rate (GFR) and progression to end-stage renal failure (ESRF) (Johnson et al., 2007).

Vascular endothelial growth factor (VEGF) is an important signalling protein involved in vasculogenesis and angiogenesis. It exerts mitogenic activity mainly towards vascular endothelial cells, although it induces effects on a number of other cell types (e.g. stimulation of monocyte/macrophage migration). The biological effect of VEGF translates through interaction with two receptors, vascular endothelial growth factor receptor 1 and 2 (VEGFR1/2). Normal function of VEGF is to create new blood vessels during embryonic development, new blood vessels after injury, and new vessels (collateral circulation) to bypass blocked vessels. When over-expressed or dysregulated, VEGF can contribute to disease pathogenesis.

The role of VEGF in renal disease and particularly in GN has been studied in a variety of experimental models and clinical studies and is incompletely understood. VEGF is an important mediator in maintaining normal kidney structure and functions (Kitamoto et al., 2002; Moeller, 2010). In addition, several lines of evidence show that up-regulation of VEGF in glomeruli may be associated with or may directly cause renal dysfunction such as in diabetic nephropathy. In a seminal study by Liu the increased expression of VEGF in glomeruli was found to directly cause glomerular hypertrophy in association with proteinuria, suggesting that VEGF exerts multiple effects on the glomerular pathophysiologic processes (Liu et al., 2007). Furthermore, increased renal tubular VEGF production in a novel transgenic mouse system has been shown to lead to fibrosis, cyst formation and glomerular disease. In this study, systemic VEGF interfered with the cross-talk between podocytes and the endocapillary compartment (Hakroush et al., 2009). In another study, participation of VEGF in glomerular capillary regeneration, crucial in the healing of GN, has been demonstrated. An analysis of glomerular VEGF mRNA and protein expression has documented a temporarily increased glomerular VEGF gene and protein expression during the healing of Habu Snake Venom model of murine mesangioproliferative GN, evoking the idea of a potential role of VEGF in the repair of mesangiolytic glomerular damage (Haas et al., 2006). In the rat models of passive Heymann nephritis and puromycin nephrosis, serial changes in glomerular capillary loop gene expression were used to uncover mechanisms contributing to primary GN. The results of the study supported the view that an early and persistent change in mostly steroid-resistant glomerular gene expression, including *VEGF* gene expression, is the hallmark of severe and progressive GN (Clement et al., 2007). In a clinical study focused on serum and urinary VEGF levels and VEGF mRNA expression in peripheral blood mononuclear cells in different GN, no difference was found in comparison to healthy controls, but a decrease in VEGF mRNA expression occurred in GN patients undergoing remission (Paydas et al., 2007).

The broad term VEGF covers a number of proteins from two families that result from alternate splicing of mRNA from a single, 8-exon, *VEGF* gene. VEGF production can be induced in cells that are not receiving enough oxygen but also by other factors, e.g. oestrogens, growth factors and cytokines. The promoter regions of the *VEGF* gene, 5' and 3' untranslated regions (UTR), are highly polymorphic and their genetic polymorphisms are supposed to influence VEGF levels, as reported in diabetic nephropathy, acute allograft rejection and some other conditions. The selection of VEGF polymorphisms at the positions -2578 (A/C) and -1154 (A/G) of the *VEGF* promoter in our study was based on their presumed effect on the serum levels of VEGF, as supported by previous studies, and also on their allele frequencies and pair-wise linkage disequilibrium coefficients. Recently, it has been suggested that CC genotype of the VEGF promoter at -2578 position might be associated with an increased risk of renal insufficiency progression in patients with IgA nephropathy (Chow et al., 2006). The aim of our study was to investigate the influence of VEGF polymorphisms at positions -2578 C/A and -1154 G/A of the *VEGF* gene promoter on the progression of selected chronic GN.

Material and Methods

Patients

The study was performed during the period 2006-2009. After the approval of the Ethical Committee, nephrologists from 22 dialysis centres and one transplantation centre in our country were asked for their co-operation. Written informed consent was obtained from all included subjects. A total of 213 patients with chronic GN (126 males, 87 females, mean age at the time of diagnosis 46.7 ± 17.1 years) entered the study. All patients were diagnosed with one of the following types of primary chronic GN: MCD/FSGS, MGN and IgAN, confirmed by renal biopsy. These GN were selected as representants of the most common primary chronic GN in adulthood, characterized by distinct pathologic and clinical features. At the time of diagnosis (which is equal to the time of the renal biopsy) and at the end of the follow-up period clinical data on arterial hypertension, serum creatinine (S-creatinine), GFR, proteinuria and serum albumin (S-albumin) were collected. Estimated GFR was assessed with the use of modification of diet in renal disease formula (MDRD formula) (Levey et al., 2006). Clinical characteristics of patients with chronic GN are summarized in Table 1. Genetically unrelated healthy Czech individuals were analysed as a control group (N = 311, 153 males, 158 females, mean age 44.6 ± 9.2 years).

Methods

Isolation of genomic DNA was accomplished by a salting-out procedure from peripheral-blood lymphocytes. Polymerase chain reaction (PCR) was performed

Table 1. Clinical characteristics of patients with chronic GN

Chronic GN	**MCD/FSGS Mean ± SD**	**MGN Mean ± SD**	**IgAN Mean ± SD**	**All GN Mean ± SD**
N	**56**	**44**	**113**	**213**
Sex (males + females)	23m + 33f n.s.	28m + 16f	75m + 38f	126m + 87f
Age at the time of diagnosis (years)	45.7 ± 20.5	56.8 ±13.9 MGN:MCD/FSGS, IgAN P < 0.001	43.2 ± 14.8	46.7 ± 17.1
Follow-up period (months)	23.7 ± 32.8 n.s.	31.4 ± 14.9	18.5 ± 28.6	23.9 ± 28.7
Arterial hypertension at the time of diagnosis	38/56 (67.9 %)	24/44 (54.5 %) MGN: MCD/FSGS, IgAN P < 0.01	87/113 (77 %)	149/213 (69.9 %)
S-creatinine$_1$ (μmol/l)	128.4 ± 131.9	85.2 ± 28.2 MGN: MCD/FSGS, IgAN P < 0.001	169.1 ± 125.3	141.1 ± 118.7
GFR$_1$ (ml/s/1.73 m^2)	1.50 ± 0.95	1.87 ± 0.91 IgAN: MCD/FSGS, MGN P < 0.001	1.10 ± 0.61	1.37 ± 0.95
proteinuria$_1$ (g/24 h) IgAN: MCD/FSGS, MGN P < 0.001	5.7 ± 4.7	5.2 ± 4.5	2.4 ± 2.4	3.9 ± 3.9
S-albumin$_1$ (g/l)	27.4 ± 9.9	24.8 ± 6.9 IgAN: MCD/FSGS, MGN P < 0.001	36.3 ± 6.7	31.6 ± 9.2
S-creatinine$_2$ (μmol/l)	126.9 ± 108.5	102.3 ± 51.3 IgAN: MCD/FSGS, MGN P < 0.001	181.7 ± 147.7	151.1 ± 127.7
GFR$_2$ (ml/s1.73 m^2)	1.41 ± 0.85	1.53 ± 0.76 IgAN: MGN P < 0.01, IgAN: MCD/FSGS: n.s.	1.15 ± 0.67	1.30 ± 0.75
Proteinuria$_2$ (g/24 h)	1.77 ± 2.85 n.s.	1.77 ± 2.86	1.18 ± 1.80	1.46 ± 2.20
S-albumin$_2$ (g/l)	39.6 ± 6.5	35.5 ± 6.8 IgAN: MCD/FSGS, MGN P < 0.001	42.6 ± 4:9	40.37 ± 6.39

S-creatinine$_1$ (serum creatinine), GFR$_1$, proteinuria$_1$, S-albumin$_1$ (serum albumin) – at the time of diagnosis
S-creatinine$_2$, GFR$_2$, proteinuria$_2$, S-albumin$_2$ – at the end of the follow-up period

using the following primers: 1) -2578 C/A polymorphism – forward primer 5'-CATGATCCCAAGCTGAAAGGCATG-3' and reverse primer 5'-GATGCTCCTGCTCTGATCC – 3', 2) -1154 G/A polymorphism - forward primer 5'-TTTCCCAGCATGTGTGTTGT-3' and reverse primer 5'-ATTTTTGTTTGTTCTCCACCGA-3'. PCR conditions were applied as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation 94 °C, annealing 56 °C and extension 72 °C. The separation of PCR products was performed in 2% agarose gel with ethidium bromide and visualization under ultraviolet light. An 18-base insertion at the -2549 position is associated with the presence of the A allele of the -2578 A/C polymorphism. A shorter allele (without insertion) is described in the presence of the C allele. The -1154 G/A *VEGF* genotyping was established after sequencing of PCR samples in an automatic ABI PRISM 310 sequencer.

Statistical analysis

For each polymorphism, allele frequencies were calculated from the genotype. The Hardy-Weinberg equilibrium was tested in the control group by the Pearson χ^2-test. The χ^2-test was used to compare the distribution of genotypes among different GN groups and among different GN groups and controls. The age of end-stage renal disease, in regard to the different genotypes, was compared by the analysis of variance (ANOVA test). Multiple comparisons using the Bonferroni method were applied. Two-sample *t*-test for comparison of two groups in submodels and paired *t*-test for testing changes during the follow-up period were used. The logarithmic transformation was applied to non-Gaussian distributed variables. The value $P < 0.05$ was considered as statistically significant.

Results

Of the overall number of 213 patients with chronic GN, the patients with IgAN were the most prevalent (Table 1). Although males were most frequent in IgAN and MGN subgroups and females prevailed in the MCD/FSGS subgroup, the sexual inequality did not reach statistical significance. The patients with MGN were older than patients with MCD/FSGS and IgAN. The mean follow-up period reached nearly two years and did not differ between diagnoses. Arterial hypertension at the time of diagnosis was present in 69.9 % of patients, the prevalence in MGN being statistically lower than in MCD/FSGS and IgAN. The renal function of the whole group of patients with chronic GN at the time of diagnosis was slightly decreased below normal range, as docu-

mented by both increased mean S-creatinine and decreased GFR. Within this group the patients with MGN had the best preserved renal functions and patients with IgAN had the most severe alteration of renal functions. Proteinuria at the time of diagnosis reached the nephrotic range in the whole group of patients with chronic GN (mean proteinuria 3.9 ± 3.9 g/24 h), being more marked in patients with MCD/FSGS and MGN than in patients with IgAN. Correspondingly, decreased levels of S-albumin were found in the whole group of patients with chronic GN, with less pronounced decrease in patients with IgAN in comparison with MCD/FSGS and MGN. Mean S-creatinine levels (151.1 ± 127.7 μmol/l) and mean GFR (1.3 ± 0.75 ml/s) at the end of the follow-up period were still decreased and did not change significantly in comparison with the initial findings in the whole group of patients with chronic GN. Within this whole group, the most decreased renal function was found in patients with IgAN. Mean proteinuria (1.46 ± 2.2 g/24 h) at the end of follow-up period was below nephrotic ranges in the whole group of patients with chronic GN and was significantly lower than at the start of the follow-up period ($P < 0.001$). The differences between the respective types of GN were not significant. S-albumin levels at the end of the follow-up period were within normal range (mean S-albumin in the whole group 40.37 ± 6.39 g/l).

The analysis of the C/A polymorphism of *VEGF* revealed an increased prevalence of CC genotype in the MCD/FSGS group in comparison with MGN and IgAN groups (Table 2). Analysis of the -1154 G/A polymorphism of *VEGF* in patients with chronic GN displayed a balanced distribution of G and A alleles between the respective types of chronic GN (Table 3). Correlation between the -2578 C/A polymorphism of VEGF and clinical and laboratory data at the start of the follow-up in MCD/FSGS showed a significant difference in mean GFR at the time of diagnosis between CC homozygotes vs. AA + AC alleles (recessive model) in favour of patients with the A allele (1.24 ± 0.64 ml/s versus 1.7 ± 1.1 ml/s, $P < 0.05$). A borderline difference between GG homozygotes vs. AA + AG alleles (recessive model) in S-creatinine at the time of diagnosis (150 ± 170.5 ml/s versus 102.4 ± 46.8 μmol/l, $P = 0.07$) was observed when analogical correlation between the -1154 G/A polymorphism of *VEGF* and clinical and laboratory data in MCD/FSGS was performed. Other data (arterial hypertension, proteinuria, S-albumin, GFR and S-creatinine at the end of follow-up) were not significantly different.

Discussion

In this study, we analysed a possible effect of two VEGF polymorphisms in the promoter of *VEGF* gene on the clinical outcome of three chronic GN. We found a higher frequency of CC genotype in the group of patients with MCD/FSGS in comparison with MGN and IgAN. Moreover, in patients with MCD/FSGS, a higher GFR at the time of diagnosis was observed in patients with AA + AC genotypes in comparison with CC homozygotes. This finding suggests a negative effect of CC genotype of the -2578 C/A polymorphism of the promoter of *VEGF* gene on the clinical course of MCD/FSGS because GFR at the time of diagnosis is considered an important prognostic factor in MCD/FSGS. Apart from this result we have not proved any significant role of both VEGF polymorphisms on the clinical course of chronic GN when GN were analysed as one group and also when the three GN were analysed separately. As far as the diagnosis of IgAN is concerned we did not confirm the association between the CC genotype of the *VEGF* promoter at -2578 position and the progression of IgAN (Chow et al., 2006). This discord-

Table 2. Distribution of the -2578 C/A polymorphism of VEGF in patients with chronic GN

	AA	AC	CC	allele A	allele C
MCD/FSGS (N = 56)	11 (19.6 %)	21 (37.5 %)	24 (42.9 %)	(38.35 %)	(61.65 %)
MGN (N = 44)	14 (31.8 %)	20 (45.5 %)	10 (22.7 %)	(54.5 %)	(45.5 %)
IgAN (N = 113)	21 (18.6 %)	62 (54.9 %)	30 (26.5 %)	(46.05 %)	(53.95 %)
All GN (213)	46 (21.6 %)	103 (48.4 %)	64 (30 %)	(45.8 %)	(54.2 %)
Control group (N = 311)	62 (20 %)	177 (57 %)	72 (23 %)	(48.5 %)	(51.5 %)

CC genotype
MCD/FSGS: MGN, IgAN $P < 0.05$
MCD/FSGS: Control $P < 0.01$
Other comparisons n.s. ($P > 0.05$)

Table 3. Distribution of the -1154 G/A polymorphism of VEGF in patients with chronic GN

	AA	AG	GG	allele A	allele G
MCD/FSGS (N = 55)	4 (7.3 %)	20 (36.4 %)	31 (56.3 %)	(25.5 %)	(74.5 %)
MGN (N = 44)	8 (18.2 %)	20 (45.4 %)	16 (36.4 %)	(40.9 %)	(59.1 %)
IgAN (N = 111)	14 (12.6 %)	45 (40.5 %)	52 (46.9 %)	(32.85 %)	(67.15 %)
All GN (210)	26 (12.4 %)	85 (40.5 %)	99 (47.1 %)	(32.65 %)	(67.35 %)
Control group (N = 311)	72 (23 %)	127 (41 %)	112 (36 %)	(43.5 %)	(56.5 %)

All comparisons n.s. ($P > 0.05$)

ant result could be explained by differences in the size of population samples and differences in the methodology used to assess the clinical significance of the VEGF polymorphism. Clearly, genetic association studies conducted so far, including our study, failed to establish an evincible link between VEGF polymorphisms at positions -2578 (A/C) and -1154 (A/G) of the *VEGF* promoter and the clinical course of selected chronic GN.

High production of VEGF has been observed both in -2578 C/C homozygote and in -1154 G/G homozygote patients (Shahbazi et al., 2002). In our previous study on VEGF polymorphisms in autosomal-dominant polycystic kidney disease (ADPKD) we observed only non-significantly increased levels of VEGF, coupled with extremely high variance of serum VEGF levels in ADPKD patients identified as CC homozygotes of the -2578 C/A polymorphism (Reiterová et al., 2008). High variance of serum production of VEGF by stimulated peripheral blood mononuclear cells and variation of VEGF in tissues could be the reason of conflicting results. It is necessary to emphasize that our knowledge of the role of VEGF in the development of GN and progression of chronic nephropathies is far from being satisfactory. If some studies underscore the association of selected VEGF polymorphisms with the susceptibility to development of primary GN (Choi et al., 2010), impairment of glomerular functions (Liu et al., 2007) or frank progression of CKD to ESRF (Summers et al., 2005), other studies highlight the essential effect of VEGF in the capillary repair of glomeruli in proliferative GN (Masuda et al., 2010). In one clinical study the decreased renal expression of VEGF was associated with worse prognosis of patients with lupus nephritis (Avihingsanon et al., 2009). In another study a decreased urinary expression of VEGF was observed in patients with severe forms of MGN (Honkanen et al., 2000). To what degree these findings apply to FSGS remains largely unknown. Down-regulation of VEGF in the glomeruli in patients with FSGS was reported by some authors (Futrakul et al., 2005; Bennett et al., 2007). On the other hand, circulating VEGF was associated with proteinuria in children with idopathic nephrotic syndrome (Cheong et al., 2001).

In conclusion, we have not confirmed any significant influence of VEGF polymorphisms at positions -2578 C/A and -1154 G/A of the *VEGF* gene promoter on the progression of chronic GN, although our study suggests a negative effect of the CC genotype of the -2578 C/A polymorphism of the promoter of the *VEGF* gene on the clinical course of MCD/FSGS.

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