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New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

Keijser, S.

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CHAPTER 10

IL-10 PROMOTOR POLYMORPHISMS ASSOCIATED WITH SUSCEPTIBILITY TO AND SEVERITY OF INFECTIOUS CORNEAL ULCERS

Submitted

S. Keijser[1], F.A.S. Kurreeman[2], R.J.W. de Keizer[1],
H. Dogterom-Ballering[3], A. van der Lelij[4], M.J. Jager[1],
P.H. Nibbering[3]

1 Department of Ophthalmology, Leiden University Medical Center, The Netherlands
2 Department of Rheumatology, Leiden University Medical Center, The Netherlands
3 Department of Infectious Diseases, Leiden University Medical Center, The Netherlands
4 Department of Ophthalmology, University Medical Center Utrecht, The Netherlands

Abstract

Purpose. In animal models for bacterial corneal ulcers, high IL-10 levels were associated with a better clinical outcome. We investigated whether IL-10 promotor polymorphisms, known to influence IL-10 expression in vitro, were associated with susceptibility to and/or clinical outcome of infectious corneal ulcers.

Methods. IL-10 promotor polymorphisms C-819T, G-1082A, A-2763C, and A-2849G were determined in 70 patients with infectious corneal ulcers and 115 healthy controls by restriction fragment length PCR analysis. For 51 patients and all healthy controls IL-10 haplotypes could be inferred using the program SNPHAP.

Results. A significant under representation of the -819C allele and A-2849A genotype were observed in the patient group compared to healthy controls, while the -2763A allele was associated with a poor clinical outcome. The IL-10.1 haplotype was associated with a poor clinical outcome, whereas haplotype IL-10.5 showed a trend towards a favorable outcome.

Conclusions. IL-10 promotor polymorphisms that are associated with low IL-10 levels seem to protect against an infectious corneal ulcer. Once a corneal ulcer has developed, IL-10 polymorphisms/haplotypes associated with a high IL-10 expression display a favorable outcome of infectious corneal ulcers.

Introduction

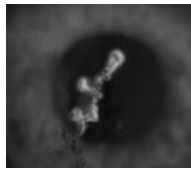
Infectious corneal ulcer can be an aggressive disease with serious complications, such as perforation of the cornea and blindness.1 Known risk factors for infectious corneal ulcers include trauma, contact-lens wear, and dry eyes.2,3 We observed in a previous study that lactoferrin gene polymorphisms may be associated with the susceptibility to and severity of infectious corneal ulcers (Keijser, submitted). However, polymorphisms in other genes may also play a role.

A major part of the damage of the cornea in infectious corneal ulcers arises from actions of the immune system itself. Anti-inflammatory mediators, such as interleukine-10 (IL-10), play a key role in preventing such excessive inflammation. The actions of this cytokine, which is produced by mononuclear phagocytes, Th2 cells and corneal epithelial cells,4 are mediated by the regulation of 1) the expression of other (proinflammatory) cytokines by a variety of cell types in the eye, 2) angiogenesis5 via various mediators, including vascular endothelial growth factor, and 3) antimicrobial defenses against e.g. Pseudomonas aeruginosa.6,7 In infectious corneal ulcers in mice, IL-10 affects the severity of the disease with high IL-10 levels being associated with a favorable outcome.8,9 In humans, different expression levels of IL-10 are related to single nucleotide polymorphisms (SNP) in the promotor region of the IL-10 gene and their haplotypes.10,11,12 These IL-10 promotor polymorphisms are involved in a variety of infections,13,14,15,16,17,18,19,20 including eye infections.21,22 Based on these considerations, we investigated whether IL-10 promotor polymorphisms are associated with the susceptibility to and/or severity of infectious corneal ulcers in man.

Patients and methods

Patients

Blood was obtained from 70 patients with an infectious corneal ulcer from the ophthalmology departments of the Leiden University Medical Center and the University Medical Center Utrecht; there were 36 male and 34 female patients, with an average age of 51 ± 20 years. Patient's records were used to extract demographic information, contact lens wear, best corrected visual acuity at onset, best-corrected visual acuity after treatment, size of the ulcer, identification of the pathogen, duration of the corneal defect, duration of antibiotic treatment, and type of treatment. Visual acuity was measured with Snellen charts. A infectious corneal ulcers was defined as an epithelial corneal defect, stromal infiltrate, purulent discharges, and with or without stromal loss. Since most ulcers resemble a circle, the size of the corneal ulcer was calculated from the diameter of the ulcer. Patients with a history of viral keratitis or a proven acanthamoeba keratitis were excluded from the study. Blood samples were available from 115 healthy unrelated control subjects (42 male, 73 female; average age 42 ± 13 years) from the same geographical region as the patients. This study was approved by the local medical ethics committee (No p03-078) and followed the tenets of the Declaration of Helsinki. All patients signed a written informed consent. From 26 patients and forty controls a tear sample was available.



Determination of IL-10 promotor polymorphisms

DNA was extracted from blood samples with the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) according to the manufacturer's instructions. IL-10 promotor polymorphisms at position C-819T, G-1082A, A-2763C, and A-2849G from the transcriptional start site were analyzed by PCR combined with restriction fragment length analysis, as previously described.[23] In brief, for C-819T and G-1082A the forward primer was: 5-CCA-AGA-CAA-CAC-TAC-TAA-GGC-TTC-TTG-AGG-A-3, and the reverse primer was: 5-AGG-TAG-TGC- TCA-CCA-TGA-CC-3. Restriction enzymes BseRI (New England Biolabs, Ipswich, MA, USA) and MslI (New England Biolabs) were used for restriction fragment length analysis of SNP C-819T and G-1082A, respectively. For SNPs A-2763C and A-2849G the forward primer: 5-TAA-AGA-AGT-CAG-ATC-CGG-GC-3, and the reverse 5-CGC-TGG-CAC-CAC-GCC-CGG-C-3 were used. Digestion was performed with restriction enzymes AlwI (Invitrogen Corporation, Carlsbad, CA, USA) for SNP A-2849G) and DdeI (Invitrogen) for SNP A-2763C.

Quality control

PCRs were run twice in order to obtain reliable data. In addition, the results were read by two independent observers. If the results of the two runs/observers differed (<1% of the cases) a third PCR was performed. All SNPs in the IL-10 promotor in the healthy control group were in Hardy-Weinberg equilibrium.

Table 1. IL-10 promotor allele and genotype frequencies among patients with an infectious corneal ulcer and healthy controls

IL-10 gene polymorphisms causing a substitution at position		Patients		Control subjects	
		Genotype frequencies	Allele frequencies	Genotype frequencies	Allele frequencies
-819	CC	47	69	63	79
	CT	44		32	
	TT	9	31*	5	21
-1082	AA	27	49	24	48
	AG	44		47	
	GG	29	51	29	52
-2763	AA	15	39	17	39
	AC	48		45	
	CC	37	61	38	61
-2849	AA	4 #	31	11	29
	AG	54		36	
	GG	41	69	53	71

A total of 70 patients and 115 healthy volunteers were involved in this study.
* p= 0.035; Chi Square test for allele frequencies
# p= 0.029; Chi-Square test for genotype frequencies

Table 2. IL-10 haplotype frequencies among patients with an infectious corneal ulcer and healthy controls

Haplotype	*SNPs*	*Patients (%)*	Healthy controls (%)
IL-10.1	CGAA	24	27
IL-10.2	CACG	18	26
IL-10.3	CGAG	8	10
IL-10.4	TACG	25	20
IL-10.5	CGCG	10	13

Haplotypes are formed by four distal SNPs in the IL-10 promotor that dictate IL-10 production. Data are from 51 patients and 115 healthy controls.

IL-10 haplotypes

From 51 of the 70 patients, haplotypes were inferred by using SNPHAP version 1.2.1 (http://www-gene.crimr.cam.ac.uk/clayton/software/). Individual haplotypes with a probability under 95% were discarded from further analysis. With SNPs C-819T, G-1082A, A-2763C, and A-2849G, the following most common haplotypes could be inferred: haplotype IL-10.1 (CGAA), haplotype IL-10.2 (CACG), haplotype IL-10.3 (CGAG), haplotype IL-10.4 (TACG), haplotype IL-10.5 (CGCG).

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Pearson Chi-Square tests were used to calculate the significance of the differences in frequencies of various SNPs in the IL-10 promotor and haplotypes between the patients and control subjects. An ANOVA-test and Cox regression analysis were used to determine the correlation between the clinical parameters among the different SNPs in the IL-10 promotor and haplotypes. P-values of 0.05 or less were considered significant. Results are expressed as odds ratios (OR) and 95% confidence intervals (CI).

Results

The allele and genotype frequencies of the four SNPs in the IL-10 promotor (C-819T, G-1082A, A-2763C, and (No p03-078), A-2849G) in patients and controls are reported in Table 1. The -819T allele was found significantly (p=0.035) more frequently in the patient group than in the control group (OR=1.68; CI=1.00-2.80). In addition, the frequency of the A-2849A genotype was significantly lower (p=0.029) in the patient group than in the control group. We calculated an OR of 0.26 (CI=0.07-0.97; p=0.045) for the A-2849A genotype compared to the A-2849G genotype and an OR of 0.52 (CI=0.14-1.99; p=0.34) compared to the G-2849G genotype.

Comparison of the various clinical characteristics defining the disease severity with the four IL-10 promotor polymorphisms revealed significantly larger corneal ulcers (p=0.014),

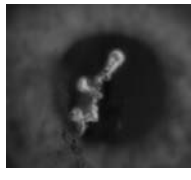


Figure 1. Association between IL-10 promotor polymorphisms -A2763C and parameters for clinical outcome. Cox regression analysis was used to calculate associations between IL-10 promotor polymorphisms -A2763C and parameters for clinical outcome.

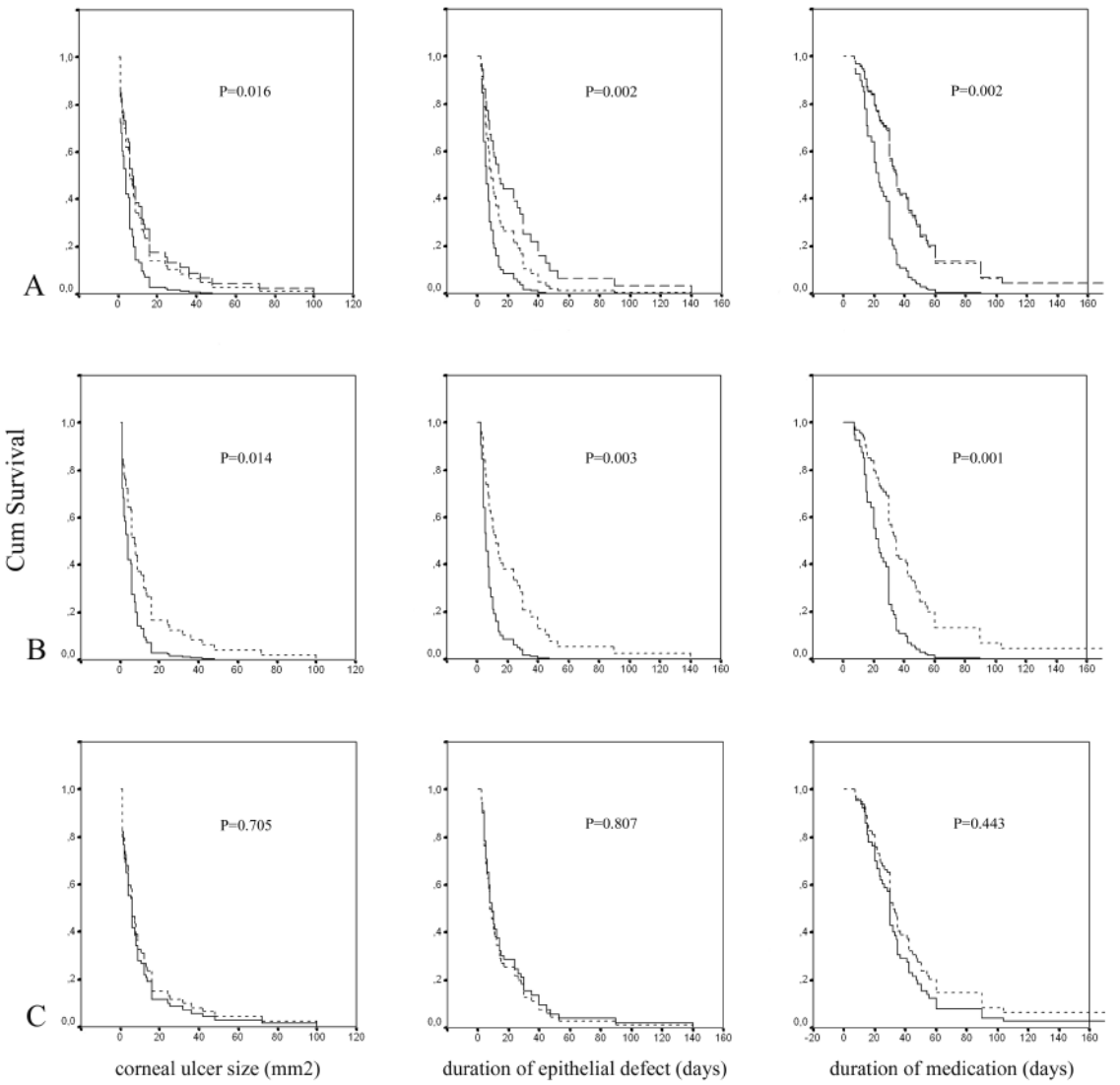
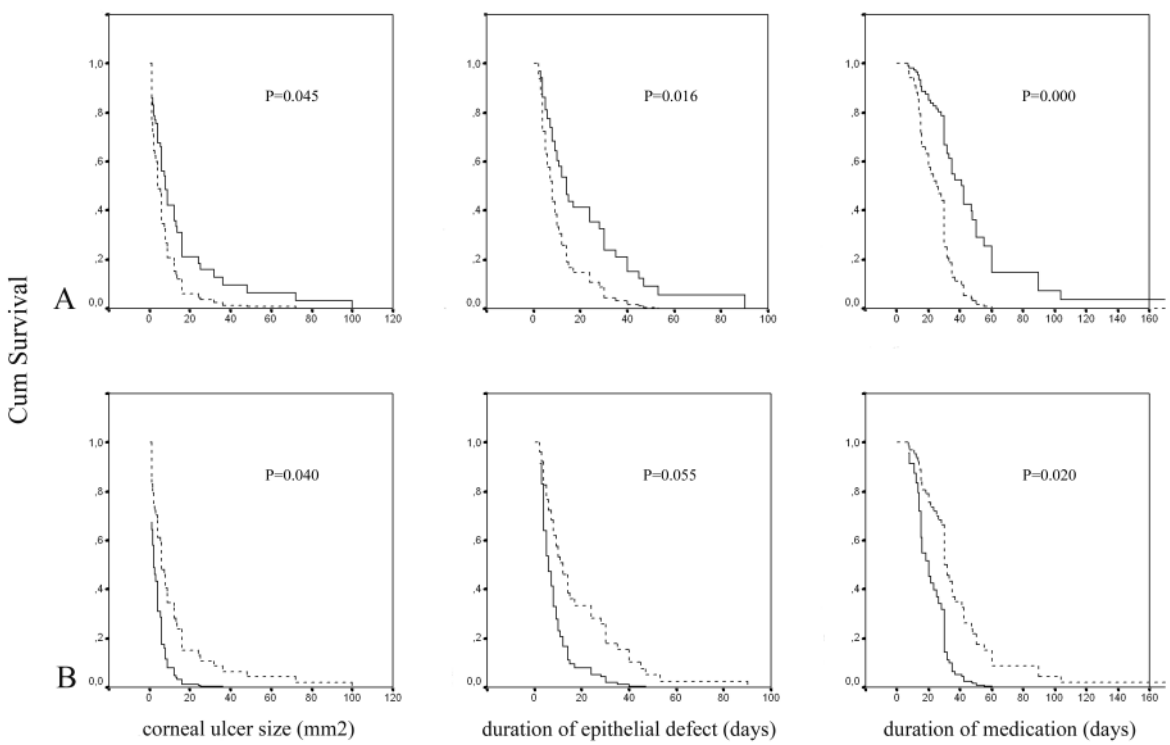
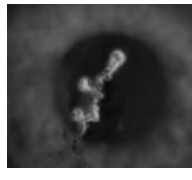


Figure 2. Associations between IL-10 haplotypes 1 and 5 and parameters for clinical severity. Cox regression analysis for associations between IL-10 haplotypes 1 and 5 and parameters for clinical severity. Note the opposite effects of the IL-10.5 and IL-10.1 haplotypes.



2A Haplotype IL10.1
—— IL10.1 present
- - - - IL10.1 not present

2B Haplotype IL10.5
—— IL10.5 present
- - - - IL10.5 not present



longer duration of the epithelial defects (p=0.003), and longer duration of treatment (p=0.001) in patients carrying the -2763A (Figure 1). Corneal ulcer size in patients carrying this allele and those carrying -2763C remained significantly different when duration of medication (p=0.024) and contact lens wear (p=0.011) were introduced as covariates in a Cox regression analysis. However, when duration of an epithelial defect was introduced as a covariate, corneal ulcer size was not significantly different (p=0.162) between patients with the -2763A and those with the -2763C allele. Furthermore, the differences in duration of the epithelial defect between patients with the -2763A allele and those with the -2763C allele remained significant when corneal ulcer size (p=0.028), duration of medication (p=0.032), and contact lens wear (p=0.005) were introduced as covariates. None of the other SNPs in the IL-10 promotor were associated with the severity of infectious corneal ulcers.

Next, we compared the various IL-10 haplotype frequencies between patients and controls and again looked for associations with disease severity. No differences were seen in haplotype frequencies between patients and controls (Table 2). With respect to the disease severity, we found that the IL-10.1 haplotype (CGAA) was associated with larger corneal ulcers (p=0.045), longer duration of epithelial defects (p=0.016), and longer duration of treatment ($p<0.001$) when compared to patients not carrying this haplotype (Figure 2). When duration of an epithelial defect was introduced as a covariate in the Cox regression analysis, no significant difference in corneal ulcer size was seen between patients carrying the IL-10.1 haplotype and those without. Duration of the epithelial defect and medication remained significantly different between these two patient subgroups when the other clinical parameters were introduced as covariates. Furthermore, the presence of the IL-10.5 haplotype was associated with smaller corneal ulcers (p=0.044), shorter duration of epithelial defect (p=0.05) and shorter duration of medication (p=0.02) in a Cox regression analysis. When the clinical parameters were introduced as covariates, only duration of antibiotic medication remained significant (p=0.045) between these two patient subgroups.

Comparison between patients with and those without contact lenses revealed a lower best-corrected visual acuity after treatment in patients without contact lenses (p=0.018) than in patients with contact lenses. All other disease severity parameters were not significantly different between patients with and those without contact lenses. Furthermore, patients with contact lenses were evenly distributed among the various IL-10 promotor genotypes and haplotypes.

IL-10 concentrations were not detectable in patients or control tear samples, both because of low volume and probably very low concentration.

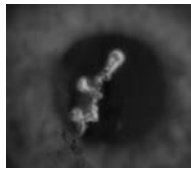
Discussion

The results of this study show that the IL-10 promotor polymorphisms C-819T and A-2849G are associated with susceptibility to infectious corneal ulcers. This conclusion is based on the differences in -819T allele and A-2849A genotype frequency between patients and healthy controls. Carriers of the -819C allele and the A-2849A genotype seem to be better protected against the development of infectious corneal ulcers. It should be realized that the A-2849A genotype is related to haplotype IL-10.1, which is associated with a low IL-10 production,12,24 whereas the -819T allele is related to haplotype IL-10.4, which is not

clearly associated with high levels of IL-10 production. These data suggest that IL-10 is important in the development of corneal ulcers but probably plays different roles in the early and late stage of corneal ulcers and/or in the defense against the different infectious agents. In this connection, it has been reported that low IL-10 levels may cause an impaired elimination of Staphylococcus aureus25 leading to destructive effects on the corneal epithelium, and Pseudomonas aeruginosa are more rapidly eliminated by high IL-10 levels.6 In agreement, others also have found different distributions of IL-10 promotor polymorphisms in infectious diseases,21,15,16,17,20 most of which are related to G-1082A. Furthermore, C-819T is associated with susceptibility to HIV infections19 and with disease severity in patients with chronic hepatitis B infections18 and those suffering from graft versus host disease.26 No relation was found between the IL-10 promotor polymorphisms C-819T and A-2849G and the disease severity in patients with infectious corneal ulcers.

Secondly, IL-10 promotor polymorphisms A-2763C are associated with disease severity; those carrying the allele -2763A suffered from a poor clinical outcome. In agreement, patients carrying haplotype IL-10.1, which comprises the -2763A allele and is associated with low levels of IL-10 in vitro,27,10 displayed a poor clinical outcome. Our data are in accordance with previous studies in mice in which an association between low IL-10 levels and an unfavorable outcome of bacterial corneal ulcers8,9,28 was seen. In addition, the IL-10 promotor polymorphisms A-2763C and IL-10.1 haplotype are associated with the duration of epithelial defect and medication. Interestingly, a trend was seen for a favourable clinical outcome in patients with IL-10.5 (Figure 2), which is related to higher transcriptional activity of the IL-10 gene.10 Although our study size is small, our observations are in alignment with earlier reports that the destruction seen in infectious corneal ulcers is partly caused by the immune system itself. High IL-10 levels could prevent excessive inflammation in response to an infection and therefore result in a less severe clinical appearance. However, replication of our findings in another study involving larger sample populations with similar clinical outcomes will shed further light on the generality of these findings. Nevertheless, it can be suggested that IL-10 therapy may be of additional value in the treatment of infectious corneal ulcers.

In conclusion, IL-10 promotor polymorphisms associated with low IL-10 levels could possibly be protective against infectious corneal ulcers, while IL-10 promotor polymorphisms associated with high IL-10 levels may regulate excessive inflammation and thereby contribute to a favourable clinical outcome.



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