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

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CHAPTER 8

LACTOFERRIN GLU561ASP POLYMORPHISM IS ASSOCIATED WITH SUSCEPTIBILITY TO HERPES SIMPLEX KERATITIS

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ABSTRACT

Lactoferrin plays an important role in the defense against infections, including herpes simplex virus (HSV) keratitis. We studied the impact of three single nucleotide polymorphisms in the human lactoferrin gene on the susceptibility to HSV infections to the eye and the severity of such infections.

Lactoferrin gene polymorphisms were determined by PCR combined with restriction fragment length analysis in 105 HSV keratitis patients and 145 control subjects. Bilateral tear samples were harvested from 50 patients and 40 healthy controls and tear lactoferrin concentrations were determined by ELISA. Patients' records were used to acquire information about the severity of the HSV keratitis.

The frequencies of the Glu561Asp polymorphism, but not those of the Ala11Thr and Lys29Arg polymorphisms, differed significantly between patients and control subjects with an under-representation of the Asp561 allele in the patient group. Furthermore, the values for best corrected visual acuity, frequency of recurrences since onset, and average duration of clinical episodes did not differ among patients with the various lactoferrin genotypes. In addition, tear lactoferrin concentrations were the same in patients with HSV keratitis and healthy controls and also did not differ among patients with the various lactoferrin genotypes.

Lactoferrin Glu561Asp polymorphism is associated with the susceptibility to HSV keratitis with a protective role for lactoferrin variants comprising Asp561. However, no beneficial effects of this lactoferrin variant on the clinical outcome of ocular HSV keratitis were noted.

INTRODUCTION

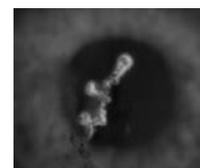
Serological studies indicated that up to 90% of the adults in the Western world have been exposed to herpes simplex virus-1 (HSV) ¹. The percentage of HSV-positive individuals rises with age and virtually all individuals over 60 years harbour HSV in their trigeminal ganglia ². However, only 17-33% of the population in the Western world suffer from HSV labialis ^{3,4,5}, and only 0.15% from ocular HSV infection ^{6,7}. Moreover, it has recently been reported that 46 out of 50 adults (92%) actively shed HSV into the tear fluid, while only 2 out of these 50 subjects had a history of ocular HSV infections ⁸. Apparently, the antiviral systems of the outer eye are highly effective in preventing the development of a clinical corneal HSV infection.

Lactoferrin, which is an important component of the non-specific defense against (HSV) infections and excessive inflammation ^{9,10}, is present at high concentrations (about 2 mg/ml)¹¹ in mucosal secretions, such as tears and milk ^{9,10,12}. It is a multifunctional protein ^{9,13} with proven efficacy against HSV ^{14,15}. In tear fluid, lactoferrin aids in the control of HSV infections by preventing HSV particles to bind to and enter epithelial cells ^{15,16,17}. Interestingly, lactoferrin also suppresses HSV infection of the mouse cornea when applied prior to the inoculum ¹⁴. Obviously, lactoferrin is an important element of the antiviral systems of the eye. Polymorphisms in the lactoferrin gene leading to amino acid substitutions at position 11 and 29 of the protein have been associated with aggressive periodontitis ^{18,19}, transcriptional activation ¹⁹, antibacterial activity ²⁰, and amino acid substitution at position 561 of lactoferrin is associated with corneal amyloidosis ²¹, and even a polymorphism located in the exon 15 not responsible for an amino acid substitution in the protein was associated with the susceptibility to diarrhea ²². Based on these considerations we investigated whether lactoferrin gene polymorphisms causing an alanine/threonine amino acid substitution at position 11 (Ala11Thr), lysine/arginine substitution at position 29 (Lys29Arg), or glutamic acid/aspartic acid substitution at position 561 (Glu561Asp) of the lactoferrin protein are associated with the susceptibility to and/or severity of HSV keratitis.

PATIENTS AND METHODS

Patients

Patients with HSV keratitis seen between 2004 and 2006 in our hospital were invited to participate in the study. Inclusion criteria were: positive diagnosis of HSV keratitis by corneal specialist, age between 18 and 90 years, and able to give written informed consent. Exclusion criteria included: an active eye infection and severe dry eyes. Of the total of 115 patients that responded to our invitation to participate in the study 105 were included. The study population consisted of 68 males and 37 females, with an average age of 56 (± 16) years. Six cases were later excluded because there was not a clear diagnosis of HSV keratitis and four because of severe dry eyes. Patients' records were used to obtain information about visual acuity at the moment of this study, number of recurrences, average duration of clinical episodes, diabetes mellitus, and contact lens wear. If the patient had been referred to our tertiary center, data on historical parameters were obtained from the patient. All patients received information, both orally and in writing, and signed an informed consent paper. From



68 patients sufficient tears from the affected eye could be collected, but from 18 of these patients no tear sample of the contra lateral eye was available. Tears were harvested with Sugi Steril sponges (Kettenbach Medical, Eschenburg, Germany), which were kept in the inferior conjunctival fornix for a few minutes²³. A blood sample (<10-ml) was available from all patients and 145 control subjects (55 ± 13 years old) without a history of severe systemic or eye infections. The characteristics of these control subjects have been described previously²⁴. Forty healthy subjects underwent a BUT and Schirmer test before a tear sample was collected. This study was approved by the local medical ethics committee (p03-078), and followed the tenets of the Declaration of Helsinki. Power analysis revealed that 80 patients and 80 healthy controls were required for this study assuming a relative risk of 2.0, and an allele frequency of 0.15, and a power of 80%.

Lactoferrin gene polymorphisms

DNA was extracted from the blood samples with the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) according to manufacturer's instructions. The DNA samples were amplified with a primer set for exon 1 and another set for exon 15 of the lactoferrin gene. Sequence for primers for exon 1 are: forward 5'-CTGTGTCTGGCTGGCCGTAGG-3' and reverse, 5'-AATGGCCTGGATACTGGAT-3', for exon 15: forward 5'-ATTCCATTGCATGGACACAG-3', and reverse 5'-CCCACACAGCTAAGAAAGCA-3'.

PCR reactions were performed in a final reaction volume of 50.16 µl comprised of 37 µl of H₂O, 3 µl of 25 mM MgCl₂ (Roche Diagnostics, Mannheim, Germany), 1 µl containing 100 ng of DNA, 5 µl of 10x concentrated PCR buffer (Roche), 1 µl containing 10 pmol of each primer (Isogen Life Science, Maarsse, The Netherlands), 1 µl of DMSO, 2 µl of 25 mM of dNTPs (Invitrogen, Carlsbad, CA, USA), and 0.16 µl of 5 U/ml Taq-polymerase (Promega®). The PCR reaction consisted of one denaturation step of 5 min at 95 °C and subsequently the PCR was done for 35 cycles: 30 sec at 95°C followed by 30 sec at 55°C and 30 sec at 72°C with a final 10 min extension at 72°C. PCR products were detected after separation on a 2 % agarose gel (Invitrogen).

To analyze the Ala11Thr, Lys29Arg, and the Glu561Asp polymorphisms, we used the following restriction enzymes: HhaI (Gibco BRL, Paisley, Scotland), MBOII (Gibco BRL), and HgaI (Biolabs, Hitchin, England), respectively. In short, the PCR product (8 µl) was mixed with 1.5 µl of 2 U/ml of restriction enzyme and 1 µl of 10x buffer and then incubated for 3 hours at 37°C. To visualize the restriction fragments, the mixtures containing HhaI or MBOII were run on spreadexgel (EL300, 50-200 bp, Elchrom, Scientific, Cham, Switzerland) and those containing HgaI on a 2% agarose gel.

ELISA for human lactoferrin

Tear lactoferrin concentrations were quantified by a human lactoferrin-specific ELISA (gift of Dr. H. van Veen, Pharming, Leiden, The Netherlands) as described by Van Berkel et al.²⁵ using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Tear samples were prediluted in PBS-1% BSA before application into the 96-well plates.

Table 1. Frequencies of genotypes/alleles encoding the amino acids at position 11, 29, and 561 in patients with herpes simplex keratitis and control subjects.

Polymorphisms		Patients		Controls	
		Genotype	Allele	Genotype	Allele
Ala11Thr	Ala/Ala	58	75	55	74
	Ala/Thr	34		37	
	Thr/Thr	8	25	8	26
Lys29Arg	Lys/Lys	54	68	49	66
	Lys/Arg	28		34	
	Arg/Arg	18	32	17	34
Glu561Asp*	Asp/Asp	7	30	16	40
	Asp/Glu	44		49	
	Glu/Glu	49	70	34	60

Data of 105 patients and 145 control subjects are expressed as percentages. All data are in Hardy Weinberg equilibrium.

* $p = 0.05$, Pearson's Chi Square test, $p=0.016$ linear by linear association.

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Pearson's Chi-Square test with 2 degrees of freedom was used to compare genotype frequencies between cases and controls. Logistic regression was used to estimate the odds-ratios of various lactoferrin genotypes between the patients and the control subjects with 95% confidence intervals. An ANOVA-test was used to determine the correlation between the clinical parameters and the different lactoferrin genotypes, and to compare the tear lactoferrin concentrations of individuals with different lactoferrin genotypes. For comparison of tear lactoferrin concentrations between the affected eye and the healthy eye in the patients group a paired samples t-test was used. P-values of 0.05 or less were considered significant.

RESULTS

To find out whether polymorphisms in the lactoferrin gene are associated with susceptibility to develop HSV keratitis, we compared the frequencies of the three lactoferrin gene polymorphisms in patients with HSV keratitis and in control subjects. The results revealed significantly different frequencies of the Glu561Asp polymorphism between patients and control subjects ($p=0.05$ by Pearson's Chi Square). A multiplicative model yields an odds ratio of 1.6 (95% CI of 1.1 to 2.5) for heterozygote individuals and an odd-ratio of 2.7 (95% CI of 1.2 to 6.1) for individuals homozygote for Asp561. No differences between patient and controls were found for the Ala11Thr and Lys29Arg polymorphisms (Table 1), both polymorphisms were in linkage disequilibrium. Next, we investigated whether the various genotypes were involved in the severity of HSV infections. The results showed no differences in best corrected visual acuity, frequency of recurrence of infection, or average duration of clinical episodes among patients with the various genotypes.

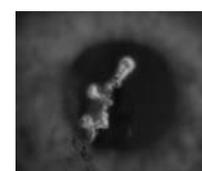


Table 2. Tear lactoferrin levels in individuals with different lactoferrin genotypes.

Polymorphisms		n	Lactoferrin concentration
Ala11Thr	Ala/Ala	39	1.6(0.1)
	Ala/Thr	21	2.0(0.4)
	Thr/Thr	5	2.2(0.6)
Lys29Arg	Lys/Lys	35	1.7(0.1)
	Lys/Arg	17	2.0(0.4)
	Arg/Arg	10	1.9(0.3)
Glu561Asp*	Asp/Asp	2	1.9(0.8)
	Asp/Glu	30	1.9(0.3)
	Glu/Glu	24	1.7(0.2)

Results are shown as mean values and SEM

Furthermore, no significant differences in tear lactoferrin concentrations among the various lactoferrin genotypes were observed (Table 2). In addition, no differences in tear lactoferrin concentrations between the affected eye and contra lateral eye of the patients (1.7 ± 0.1 mg/ml and 1.8 ± 0.2 mg/ml, respectively; $n=50$) were found, and these values were similar to the tear lactoferrin concentrations of the right and left eye of healthy controls (1.9 ± 0.2 mg/ml and 2.1 ± 0.2 mg/ml, respectively; $n=40$). No differences in clinical parameters were observed between patients from whom a tear sample was available ($n=68$) and patients from whom a tear sample was lacking ($n=37$).

DISCUSSION

We conclude from this study that the lactoferrin Glu561Asp polymorphism is associated with the susceptibility to HSV keratitis, but not with the severity of the disease or frequency of its recurrences. This is based on the following findings. First, the frequencies in the Glu561 and 561Asp alleles, but not those of the Ala11Thr and Lys29Arg lactoferrin polymorphisms, differed significantly between patients and control subjects. These data are in accordance with an earlier report that genetic variation plays an important role in the development of ocular herpetic disease²⁶. Secondly, no correlation was found between the various lactoferrin genotypes and the severity of the disease, i.e. best corrected visual acuity, average duration of clinical episodes, and frequency of recurrent infections.

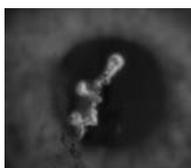
Although our data is derived from a relatively small number of patients, the present number of patients in the present study was higher than that calculated by the power analysis performed at the start of the study. To confirm the association between Glu561Asp polymorphism and HSV keratitis susceptibility our study needs to be replicated in a different, larger cohort. Nevertheless, our data indicates that individuals with lactoferrin comprising Asp561 are less susceptible to HSV keratitis than those with the lactoferrin variant with Glu561. Of note, the lack of positive effect of Glu561 on the frequency of recurrence in HSV patients can be explained by the different ports d'entrée of the virus. In a primary infection the HSV

is thought to infect the corneal epithelium from the outside, i.e. through the lactoferrin containing tear film, while in recurrent disease the HSV is reactivated from its latent state in the trigeminal ganglia and can infect the cornea through the trigeminal nerve fibers in the stroma of the cornea, without any involvement of tear lactoferrin.

The mechanisms by which the Glu561Asp polymorphism can influence the primary ocular HSV infection are not known. The amino acid at position 561 in lactoferrin locates in a loop region at the bottom of the C-lob. The oxygen of the Glu561 side chain may form a weak hydrogen band with the nitrogen of the Trp563 side chain, while the Asp561 lacks this possible hydrogen bond. This may enhance the flexibility of the Asp561 lactoferrin variant compared to the Glu561 variant leading to the exposure of a hydrophobic domain to the environment²¹. Whether this exposed hydrophobic domain contributes to the antiviral activities of lactoferrin needs to be investigated. For this purpose recombinant lactoferrins varying at position 561 will be prepared and their antiviral activities analyzed using established assays. The lack of association between Ala11Thr and Lys29Arg polymorphisms and susceptibility to HSV keratitis together with the association between Glu561Asp polymorphisms and disease susceptibility indicates that the hydrophobic region comprising amino acid 561 affects the antibacterial and antiviral activities differently.

Furthermore, our observation that the tear lactoferrin concentrations did not differ among the various genotypes encoding amino acids at the three positions in the lactoferrin protein excludes the possibility that the protective role of the Asp561 allele is mediated by higher tear lactoferrin levels in these patients. Finally, the tear lactoferrin concentration did not significantly differ between patients and control subjects, which is an interesting finding because in vitro studies have found an influence of the lactoferrin concentration on HSV infections^{14,15}.

To elucidate genetic determinants of susceptibility to eye infections and/or the severity of these diseases, we first analyzed the association of lactoferrin gene polymorphisms with the susceptibility and/or severity of HSV keratitis. We realize that HSV keratitis is a complex process involving virulence factors of the virus, such as glycoproteins B,C, D, H and L, as well as host molecules, including heparan sulfates and the entry receptors herpes virus entry mediator (HVEM) and nectin-1 and-2^{27,28} cytokines like IL-10²⁹, IL-12³⁰, and INF- γ ^{31,32}, and glucocorticoids³³, and antimicrobial proteins/peptides, such as human alpha- and beta-defensins,³⁴ cathelicidins³⁵ and lactoferrin/lactoferricin²⁸. Obviously, polymorphisms in the genes encoding these host factors (or their receptors and/or signalling molecules) may also contribute to the susceptibility and/or the severity of HSV keratitis.



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