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CYTOKINE POLYMORPHISMS IN MEN WITH CHRONIC PROSTATITIS/ CHRONIC PELVIC PAIN SYNDROME: ASSOCIATION WITH DIAGNOSIS AND TREATMENT RESPONSE

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ABSTRACT

Purpose: The chronic pelvic pain syndrome is a common disorder of unknown etiology. Elevated cytokines in prostate fluid and semen are frequent findings. We studied genetic polymorphisms that can alter cytokine gene expression in men with the chronic pelvic pain syndrome.

Materials and Methods: Genomic DNA was extracted from blood from 36 men with the chronic pelvic pain syndrome. Reversed sequence specific oligonucleotide probing was used to genotype the polymorphisms for cytokine promoter sites, namely tumor necrosis factor (TNF)-α 308, transforming growth factor (TGF)-β 25, TGF-β 10, interleukin (IL)-10 1082 and IL-6 174. Genotype frequencies were compared with 252 controls as well as among groups of patients with the chronic pelvic pain syndrome according to diagnostic category and treatment response.

Results: There were no differences in men with the chronic pelvic pain syndrome and control patients in the frequency of TNF-α, TGF-β or IL-6 alleles, although those with the chronic pelvic pain syndrome were more likely to express the genotype associated with low IL-10 production (30.6% versus 12.1%, $p = 0.007$). When comparing National Institutes of Health diagnoses, category IIIa patients were more likely to have the low TNF-α genotype (categories II, IIIa and IIIb 33%, 100% and 18%, respectively, $p = 0.04$). All 11 of the 28 patients treated with the anti-inflammatory quercetin in whom treatment failed had the low TNF-α genotype versus 29.4% of those in whom treatment succeeded ($p = 0.0003$). Similarly men with quercetin treatment failure were much less likely to have the low IL-10 genotype than those with treatment success (9.1% versus 47.1%, $p = 0.04$).

Conclusions: Patients with the chronic pelvic pain syndrome are more likely to have a low IL-10 producing genotype, suggesting autoimmunity as a potential etiology. Anti-inflammatory phytotherapy failure was associated with low TNF-α and high IL-10 phenotypes, which may help define a subset of patients with the chronic pelvic pain syndrome without an inflammatory etiology.

KEY WORDS: prostate, prostatitis, cytokines, autoimmunity, gene expression

Men with National Institutes of Health (NIH) category III prostatitis (chronic pelvic pain syndrome) do not have urinary tract infections but have the typical symptoms of pain and urinary voiding dysfunction. The etiology of this disorder is controversial with groups postulating infectious, autoimmune, inflammatory or neuromuscular mechanisms. A study showed abnormal levels of inflammatory cytokines in expressed prostatic secretions or semen in men with the chronic pelvic pain syndrome.[1] Polymorphisms have been identified in the promoter regions of several cytokines, of which the genotypes confer differential expression of their respective cytokines from similar stimuli. Therefore, a hypothesis is that men in whom the chronic pelvic pain syndrome develops based on inflammation or autoimmunity may have a genetic predisposition for altered cytokine expression. For example, men more likely to have autoimmune chronic pelvic pain syndrome may produce high levels of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α or interleukin (IL)-6, or low levels of immune suppressive cytokines, such as transforming growth factor (TGF)-β or IL-10. Furthermore, if different etiologies can lead to the chronic pelvic pain syndrome, men with different diagnoses or response to specific treatments may be more likely to express different cytokine genotypes.

We determined the genotype in men with the chronic pelvic pain syndrome for several proinflammatory and anti-inflammatory cytokine genes. The frequency of each genotype was compared with frequencies in a control population to determine general predisposing factors. Genotype frequencies were then compared among patients with the chronic pelvic pain syndrome based on diagnostic category (II versus IIIa versus IIIb) and response to treatment.

METHODS

A 5 ml. sample of whole blood was collected from 36 men attending a specialty chronic prostatitis clinic between January and July 2001. Results were compared with 252 samples from control patients, including kidney transplant donors and recipients. There were no appreciable differences in polymorphism percents in our control population and published control studies. All men with the chronic pelvic pain syndrome had had pain and/or urinary symptoms for at least 3 of the last 6 months and did not have a urinary tract infection during this time. Men with previous prostatic surgery (other than needle biopsy), malignancy or chlamydial urethritis were excluded from analysis. All patients underwent a complete history and physical examination, including

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the NIH Chronic Prostatitis Symptom Index (CPSI), urethral/urine culture and culture of expressed prostatic secretions. The white blood count in expressed prostatic secretions was considered the mean of 4 high power fields. Men with established uropathogens (for example Escherichia coli or enterococcus) that localized to the expressed prostatic secretions were classified as category II. The remaining category III patients were classified as IIIa if the expressed prostatic secretion white blood count was 10 cells per high power field or greater, while, otherwise they were classified as IIIb.

After the initial visit patients received various therapies, including antibiotics, prostatic massage, α-blockers, antiinflammatories, neuromuscular agents or physiotherapy. Patient response to therapy was considered positive if most or all of their symptoms were resolved and the patient did not require any further or additional therapy to control symptoms. It was typically associated with a greater than 50% improvement in total NIH-CPSI score, although there were insufficient patients with a final score for meaningful statistical analysis. Laboratory analyses were performed by investigators blinded to patient names and diagnoses. Genomic DNA from frozen whole blood samples was obtained using Qiagen columns (Qiagen, Inc., Valencia, California) according to the manufacturer procedure.

Primers and probes used in this study were synthesized in our laboratory using the ABI Expedite nucleic acids synthesis system (Applied Biosystems, Foster City, California). Primers were biotinylated using biotin teg phosphoramidite (Glenn Research, Sterling, Virginia). Table 1 lists all polymerase chain reaction (PCR) primers, annealing temperatures and predicted amplicon sizes. Each 100 μl. PCR reaction mixture contained 10 μl. genomic DNA (200 to 500 ng.), 10 μl. 10 $\times$ deoxynucleotide triphosphates (Life Codes Corp., Stamford, Connecticut), 10 μl. 10 $\times$ PCR buffer (Life Codes), 16 μl. 6 M. betaine monohydrate (Sigma Chemical Co., St. Louis, Missouri), 2 μl. of each biotinylated primer (20 μM. stock), 4 units Taq polymerase (Gibco BRL, Grand Island, New York) and 50 μl. water. Amplification reactions were performed on a PE 9600 thermocycler (Perkin Elmer, Boston, Massachusetts) starting with an initial melting time of 3 minutes, followed by 30 cycles with each cycle consisting of 30 seconds at 95C, annealing temperature for 30 seconds (table 1), extension for 45 seconds at 72C and final extension for 7 minutes at 72C. Amplified products were visualized on 2% agarose gel with ethidium bromide using an ultraviolet light box.

Genetic screening using sequence specific oligonucleotide probes. After PCR reactions specific biotinylated amplicons for each cytokine gene were produced. To screen and genotype these amplicons a high throughput plate based genetic screening method was used. Table 2 lists the probes used in this study. These probes were enzymatically t tailed using terminal deoxynucleotidyl transferase (TdT) (Boehringer-Mannheim, Indianapolis, Indiana). Briefly, 1 nmol. of each probe was used in a 240 μl. reaction mixture that contained 48 μl. 5 $\times$ TdT buffer (Boehringer-Mannheim), 5 μl. 100 mM. deoxythymidine triphosphate, 8 μl. (200 units) TdT and 1 μl. pyrophosphatase enzyme (Sigma Chemical Co.). The tailing reaction was incubated for at least 4 hours and stopped with 60 μl. 0.5 M. ethylenediaminetetraacetic acid, followed by adding 700 μl. tris-edetic acid buffer. This protocol resulted in adding a homopolymer tail of approximately 400 bases per probe, as estimated from the 15% polyacrylamide tris-borate-edetic-acid-urea gel (Novex, San Diego, California).

TABLE 1. *Cytokine primers, melting temperatures and predicted amplicon sizes*

Primer	Sequence	Melting Temperature (C)	Size (bp)
IL-10:			
Sense	5′-ATCCAAGACAACACTAA-′3	55	528
Antisense	5′-TAAATATCCTCAAAGTTCC-′3		
TNF-α:			
Sense	5′-ACTCAACACAGCTTTTCCCTCCA-′3	64	260
Antisense	5′-TCCTCCCTGCTCCGATTCCG-′3		
TGF-B1:			
Sense	5′-CTTCACCAGCTCCATGTCGATAG-′3	60	274
Antisense	5′-ACTGCGCCCTTCTCCCTG-′3		
IL-6:			
Sense	5′-GCCTGTTAATCTGGTCACTGAA-′3	60	412
Antisense	5′-GCCTCAGACATCTCCAGTCC-′3		

TABLE 2. *Oligonucleotide probes used to identify gene cytokine polymorphism*

Probe	Sequence	Melting Temperature (C)
TGF-B1:		
Codon 10 C	5′-GCTGCTGCCGCTGCTGC-′3	60
Codon 10 T	5′-GCTGCTGCTGCTGCTGC-′3	
Codon 25 G	5′-GCCTGGCCGGCCGGCCG-′3	66
Codon 25 C	5′-GCCTGGCCCGCCGGCCG-′3	
TNF-α:		
-308 G	5′-GAGGGGCATGGGGACGG-′3	62
-308 A	5′-GAGGGGCATGAGGACGG-′3	
IL-10:		
-1082 G	5′-TTCTTTGGGAGGGGGAAG-′3	56
-1082 A	5′-TTCTTTGGGAAGGGGAAG-′3	
IL-6:		
-174 G	5′-GTGTCTTGCGATGCTAAAGG-′3	60
-174 C	5′-GTGTCTTGCCATGCTAAAGG-′3	

Immobilizing t tailed probes on microtiter plates. A specific t tailed probe (1 pmol.) in 100 μl. tris-edetic acid buffer was added to each well of 96-well Costar plates (Corning Costar, Badhoevedorp, The Netherlands). Subsequently the plates were incubated overnight in a 45C drying oven, removed, blocked with a blocking buffer (5 $\times$ Denhart's in phosphate buffered saline) for 4 hours, air dried sealed with a desiccant pouch and stored at 4C.

Hybridization and detection. Hybridization temperatures were adjusted according to the individual probe (table 2). Each microtiter plate consisted of 1 specific probe sequence and, therefore, would identify 1 polymorphic site. Denaturing solution (5 μl. 0.25 N NaOH) was dispensed in each well, followed by the addition of 5 μl. biotinlyated amplicon. After allowing the DNA to denature at room temperature for 5 minutes 100 μl. hybridization solution, consisting of sodium phosphate, 5 $\times$ Denhart's and 0.2% sodium dodecyl sulfate (SDS), were added and the plates were incubated in a hot air incubator at the appropriate temperature for 60 to 90 minutes. At that point 200 μl. washing solution (3 M. tetramethylammonium chloride/0.1% SDS, Life Codes Corp.) were added and the plates were incubated at 61C for 10 minutes (except TGF-β codon 25, which was incubated at 66C for 20 minutes). The plates were washed 3 times with 200 μl. 1 $\times$ washing solution (0.5 $\times$ saline sodium citrate and 0.1% Tween 20) at room temperature. Subsequently 100 μl. conjugate diluent (5 $\times$ saline sodium citrate, 5 $\times$ Denhart's and 0.1% SDS) that contained streptovidin horseradish peroxidase conjugate, diluted 1:100,000, was added and the plates were incubated 15 minutes at 37C. Streptovidin horseradish peroxidase was removed by washing the plated 4 times with 1 $\times$ washing solution at room temperature. Subsequently 100 μl. 1.25 mM. tetramethylbenzidine (Sigma Chemical Co.) substrate solution was added and incubated (if needed) at 37C for 10 minutes. After blue color development, which indicates a positive result, the reaction was stopped by adding 100 μl. stop solution (0.5 M. HCl) and the plates were read on an enzyme-linked immunosorbent assay plate reader at 450 nm.

Statistics. Statistical comparisons were performed using the chi-square or Fisher exact test if the expected incidence within a cell was less than 5. Significance was considered at

$p < 0.05$. If $p < 0.1$ the exact value is stated but otherwise it is expressed as not significant.

RESULTS

In the 36 men with the chronic pelvic pain syndrome average age was 42 years (range 24 to 77) (table 3). They had symptoms of the chronic pelvic pain syndrome for a mean of 6.9 years (median 2.5, range 6 months to 26 years). Mean NIH-CPSI component values plus or minus standard deviation were pain 9.5 ± 3.5, urinary 4 ± 2.9, quality of life 8.6 ± 2.4 and total score 21.8 ± 6.7. Symptomatic categories were typical for men with this condition since 97% had pain, 58% had irritative voiding symptoms, 56% had obstructive voiding symptoms and 39% had erectile dysfunction. Based on cultures of urethra, urine and expressed prostatic secretions 6 patients had uropathogens that localized to the prostate (NIH category II). Of the remaining patients 8 had greater than 10 white blood cells per high power field in expressed prostatic secretions (category IIIa), while 20 did not (category IIIb). The mean white blood count in expressed prostatic secretions was 6.7 ± 6.0 cells per high power field (range 0 to 20) and the mean count in IIIa and IIIb patients was 14.2 and 3.1, respectively.

Table 4 shows the relative genotype frequencies of each cytokine promoter polymorphism. There were no differences in gene frequencies in patients with the chronic pelvic pain syndrome and controls for TNF-α, the TGF-β gene or IL-6. A significantly higher proportion of patients with the chronic pelvic pain syndrome expressed the IL-10 AA genotype, which is associated with low IL-10 expression, than controls (30.6% versus 12.1%, $p = 0.007$).

Based on culture results and microscopic white blood count in expressed prostatic secretions there were 6 category II (bacterial infection), 10 category IIIa (nonbacterial 10 or greater white blood cells per high power field) and 22 category IIIb (nonbacterial less than 10 white blood cells per high power field) patients. Table 5 shows the proportion of patients according to category who expressed the genotype associated with low cytokine expression. All category IIIa patients had the low TNF-α genotype ($p = 0.04$) and none of the category II patients had the low IL-6 genotype ($p = 0.29$).

The data were then analyzed according to demographic and clinical criteria. No differences in cytokine polymorphisms were found based on age at onset, presence or absence of particular symptoms, symptom severity based on the NIH-CPSI, response to antibiotic therapy or α-blockers. Of the 28 men treated with the anti-inflammatory quercetin supplement Prosta-Q (Farr Laboratories, El Segundo, California) there were differences in the 11 who did not and the 17 who did improve. Table 5 shows that 100% of the patients in whom this therapy failed had the GG TNF-α genotype (low cytokine expression) versus 29.4% of responders ($p = 0.0003$), while only 9.1% of those with treatment failure had the low IL-10 genotype versus 47.1% of responders ($p = 0.04$).

TABLE 3. *Characteristics of study patients*

	Mean ± SD	Median (range)
Age	42 ± 11	43 (24–77)
Symptom duration (yrs.)	6.9 ± 7.6	2.5 (0.5–26)
No. white blood cells/high power field in expressed prostatic secretions	6.7 ± 6.0	5 (0–20)
NIH-CPSI score:		
Pain	9.5 ± 3.5	9.5 (0–16)
Urinary	4 ± 2.9	4 (0–10)
Quality of life	8.6 ± 2.4	10 (4–13)
Totals	21.8 ± 6.7	20.5 (11–36)

TABLE 4. *Genotype incidence in study group and general population*

	Chronic Pelvic Pain Syndrome	Controls	p Value
No. pts.	36	272	
% TNF-α 308:			
GG	61.1	69.0	
GA	33.3	24.4	Not significant
AA	2.8	5.4	
% TGF-β 25:			
CC	8.3	5.4	
CG	22.2	29.3	Not significant
GG	69.4	65.3	
% TGF-β 10:			
CC	2.8	16.3	
TC	58.3	45.5	Not significant
TT	36.1	37.8	
% IL-10 1082:			
AA	30.6	12.1	0.007
GA	33.3	62.5	
GG	36.1	22.8	
% IL-6 174:			
CC	15.2	11.8	
CG	54.5	58.8	Not significant
GG	30.3	26.1	

DISCUSSION

The chronic pelvic pain syndrome is a common and perplexing disorder affecting about 9% to 14% of men worldwide.[2] In patients with negative cultures the etiology and optimal treatment have been controversial. Molecular techniques demonstrate the presence of bacteria in the prostatic fluid of some men with negative cultures and in fact many with the chronic pelvic pain syndrome have some improvement with antibiotic therapy. Many patients have negative cultures and minimal response to antibiotics, and several lines of evidence suggest an inflammatory or true autoimmune condition. Men with the chronic pelvic pain syndrome have elevated oxidative stress in expressed prostatic secretions[3] and semen. Elevated cytokines have been documented in seminal fluids of patients with the chronic pelvic pain syndrome for TNF-α,[4] IL-6,[5] IL-8[6] and IL-1β.[7] Importantly no study has shown a correlation of the measured white blood count with cytokine levels, suggesting that it is not the number of visible inflammatory cells that defines inflammation but their activity and products.

There is increasing evidence that cytokine genes are polymorphic and some of these polymorphisms are associated with altered levels of cytokine expression in vivo. Susceptibility to numerous infectious and autoimmune conditions have been associated with particular cytokine genotypes. Cytokine polymorphisms associated with disease processes include TNF-α (asthma,[8] ankylosing spondylitis,[9] primary sclerosing cholangitis[10] and inflammatory bowel disease[11]), IL-10 (inflammatory bowel disease[11,12]), IL-6 (juvenile arthritis[13]) and IL-1 (periodontitis[14] and Alzheimer's disease[15]).

In this study we examined the frequency of known polymorphisms of 2 proinflammatory cytokines (TNF-α and IL-6) and 2 anti-inflammatory cytokines (TGF-β and IL-10). Compared with a control population the only significantly different proportion of genotype expression was that of IL-10. A significantly higher proportion of patients with the chronic pelvic pain syndrome had the AA genotype (30.6% versus 12.1% of controls), which is the genotype associated with decreased expression of IL-10.[16] Since high IL-10 expression produces anti-inflammatory effects,[12] this association may suggest that men with the chronic pelvic pain syndrome are predisposed to inflammatory/autoimmune conditions. Experimental rodent models of autoimmune prostatitis have long been known,[17] although their relevance for symptomatic chronic prostatitis has never been established. Alexander et

TABLE 5. *Incidence of low expression genotype by prostatitis category and gene polymorphism by anti-inflammatory treatment response*

	No. Pts.	% TNF-α GG	% IL-10 AA	% IL-6 CC
Category:				
II	6	33.3	16.7	0
IIIa	8	100	12.5	12.5
IIIb	22	18.2	40.9	18.2
p Value		0.04	Not significant	Not significant
Treatment success:				
No	11	100	9.1	18.2
Yes	17	29.4	47.1	11.8
p Value		0.0003	0.04	Not significant

al demonstrated in vitro evidence for T-cell proliferative responses to seminal antigens in 3 of the 10 patients with the chronic pelvic pain syndrome studied.[18] In fact, there is a case report of resolution of the chronic pelvic pain syndrome in a completely immunosuppressed transplant patient.[19] In men with low IL-10 production autoimmune prostatitis may be more likely to develop after prostatic injury (with unmasking of hidden antigenic epitopes) or direct T-cell stimulation from gram-positive bacteria such as staphylococci, which can induce autoimmunity through superantigen[20] and are commonly found in the prostatic fluid of men with the chronic pelvic pain syndrome.[21] Conversely men with high IL-10 production may be able to respond to such injury or infection with a more controlled inflammatory response that does not persist after the initial injury is controlled.

Few differences in gene expression were detected among diagnostic groups of patients. None of the patients with prostatic infection had the genotype associated with low IL-6 expression. High IL-6 levels have been associated with chronic prostatitis that improves with antibiotic therapy.[22] However, due to small numbers in our groups more patients would require testing to establish a true statistical association. All patients in category IIIa had the genotype associated with low TNF-α production compared with 20% of IIIb patients. It may be postulated that men with low TNF-α responses have longer and sustained induction of white blood cell attracting chemokines because TNF-α induces IL-10 production[23] and low IL-10 production is proinflammatory. More white blood cells, especially neutrophils, may then be recruited to within the prostatic acini. The true significance of the white blood count in expressed prostatic secretions is questionable whether measured by wet mount or with a more accurate counting chamber. In men with the chronic pelvic pain syndrome white blood counts fluctuate with time[24] and white blood counts do not correlate with symptom severity, levels of oxidative stress[3] or seminal cytokine levels.[5] It is likely that these markers of inflammation are more important diagnostic criteria than white blood counts.

Of the men in this study 28 were treated with the anti-inflammatory herbal supplement Prosta-Q containing the bioflavonoid quercetin. Quercetin has been shown to be clinically effective in men with category III chronic prostatitis.[25] Therapy for 1 month lowers levels of oxidative stress[3] and prostaglandin E2[26] in expressed prostatic secretions. Based on these mechanisms one would expect that patients with the most inflammatory/autoimmune profiles would benefit most from this therapy and those with no inflammation would benefit the least. In the patients who significantly improved with quercetin therapy there was no identifiable pattern of cytokine genotypes. However, all 11 patients who did not improve with quercetin had the low TNF-α expression genotype but only 9.1% had the low IL-10 expression genotype. Therefore, those with quercetin failure had the least inflammatory genotype. While empirical antibiotic therapy may identify infection as the underlying cause of the chronic pelvic pain syndrome in patients with infection (although antibiotics can have direct anti-inflammatory effects), clinical response to quercetin therapy may help stratify patients with or without inflammatory prostatitis more accurately than conventional white blood cell measures.

It is important to realize the limitations of polymorphism studies such as ours. While clear correlations were noted among the cytokine polymorphisms described in this study for TNF-α[27] and IL-10,[28] there is no guarantee that these relationships necessarily hold true for individuals. A patient with a low cytokine producing genotype may nevertheless produce large quantities of its cytokine given the appropriate strong stimulus. By analogy many patients with autoimmune arthritis have the HLA-B27 allele but in most with the HLA-B27 allele arthritis does not develop. Nevertheless, genotypic trends can suggest etiological pathways for cryptic disorders such as the chronic pelvic pain syndrome and help stratify patients who may benefit the most from individual therapies. A genetic predisposition for inflammatory responses in men with the chronic pelvic pain syndrome may also help explain the frequent association with other conditions, such as sinusitis, arthralgias, the chronic fatigue syndrome[29] and irritable bowel disease (unpublished data).

CONCLUSIONS

We studied cytokine polymorphisms in 36 men with the chronic pelvic pain syndrome and found a higher than expected proportion of the allele associated with low IL-10 expression, which suggests a proinflammatory state. Category IIIa patients (nonbacterial prostatitis) were more likely to express the low TNF-α genotype. Patients who did not improve with anti-inflammatory quercetin therapy had a noninflammatory genotype (low TNF-α and high IL-10). Together these results add further evidence that some patients with chronic prostatitis have a predisposition for autoimmune/inflammatory disorders and this subset may best be targeted with anti-inflammatory therapies.

APPENDIX

For a current comprehensive online database of cytokine genotypes see http://bris.ac.uk/pathandmicro/services/GAI/cytokine4.htm.

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