Abstract

**Purpose:** Chlamydia pneumonia is a known endothelial pathogen in several vascular diseases including atherosclerosis, age-related macular degeneration and non-arteritic anterior ischemic optic neuropathy. Diabetic retinopathy is a retinal vascular disease that may similarly be affected. We serologically investigated the presence of C. pneumonia infection in patients with proliferative diabetic retinopathy.

**Design:** Pilot case-control study.

**Patients & Methods:** During a period of 12 months, we examined sera of 27 type I diabetic patients with different stages of diabetic retinopathy for anti-chlamydial IgG and IgA by enzyme-linked immunosorbent assay (ELISA).

**Results:** The IgG seropositivity was significantly different between different diabetic retinopathy groups (P=0.017). Rate of seropositivity was higher among patients with PDR comparing to patients without PDR, with a marginal significance (P=0.062). Although the titer of IgA was negative in all patients (less than 0.357 U/mL), the IgA titer was higher in patients with PDR than those without PDR, reaching the significance at P<0.06 level.

**Conclusion:** C. pneumonia may be an independent risk factor for proliferative diabetic retinopathy.

**Key words:** Chlamydia Pneumonia, Diabetic Retinopathy, Serology, Diabetes Mellitus

Introduction

Diabetic eye disease, in particular diabetic retinopathy, is one of the most common causes of blindness in the western world. It remains a major cause of blindness in patients aged 20-64 years. From an ophthalmic point of view, proliferative diabetic retinopathy (PDR) and its complications is a major problem. In general, the majority of diabetics can be expected to develop retinopathy with time and 30–50% can be expected to develop sight-threatening retinopathy, namely proliferative retinopathy or diabetic macular edema, in their lifetime. Preventing PDR, not only saves the vision, but also offers patients a higher chance for better disease control.

There are a number of known risk factors for progression of diabetic retinopathy, including longer duration of diabetes, younger age at diagnosis, presence of diabetic neuropathy, high HbA1c level, decreased hematocrit, increased serum triglyceride levels, and decreased albumin.

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However, other risk factors may play an important role in the disease process. Atherosclerosis deserves special attention as it is a predisposing factor for other vasculopathies. Interestingly, some of the risk factors for PDR are also the predisposing situations for atherosclerosis, so atherosclerosis may be an intermediate risk factor for PDR. Infectious causes are under active investigations as contributing factors in the pathogenesis of atherosclerosis. Among various microorganisms, including measles virus, herpes simplex virus, cytomegalovirus, Helicobacter pylori, Corynebacterium diphtheriae, and Chlamydia pneumonia, the last agent seems to be a more important pathogen in the formation of atheromatous plaques. Further more, C. pneumonia is implicated in a number of ocular diseases, including acute anterior uveitis, age-related macular degeneration (ARMD), age-related macular degeneration (ARMD), anterior ischemic optic neuropathy (AION), and central retinal vein occlusion (CRVO). Regarding the probable common pathogenic pathways, investigation of the possible role of C. pneumonia in the pathogenesis of diabetic retinopathy is noteworthy.

Materials and Methods

After being approved by the research and ethical board of Mashhad University of Medical Sciences, the study was conducted in Khatam al-Anbia eye hospital from March 2004 to March 2005. This is a tertiary, referral hospital in Northeast of Iran. Patients included in the study were type I diabetics who were 15-30 years old at the time of diagnosis of diabetes and were less than 55 years at the time of enrolment. All of patients had diabetes for more than a year. Written informed consent forms were signed by all participants. Those with known history of coronary artery diseases, cerebrovascular attacks, peripheral vascular disorders, and respiratory tract infections were excluded. Patients meeting the research criteria had a complete ophthalmic exam and a fresh sample of blood was sent for serologic evaluation to the Immunology Research Center of the university. All of the specimens were referred to with a randomized coding system and the examining ophthalmologist and laboratory personnel were blind to the results. The stage of diabetic retinopathy was classified according to the Early Treatment of Diabetic Retinopathy Study (ETDRS) classification. Accordingly patients were sub-classified to those having PDR in at least one eye and those who do not have PDR. The latter “non-PDR” group composed of “no diabetic retinopathy” (NDR) and “non-proliferative diabetic retinopathy” (NPDR) patients and patients with non-proliferative diabetic retinopathy in one eye and no diabetic retinopathy in the other eye. Patients' data, including age, sex, history of hospital admission due to diabetic ketoacidosis, concomitant diseases and presence or history of diabetic microangiopathy including neurologic problems and dermatologic diseases such as shin spots or necrobiosis lipoidica diabetorum, insulin dosage, and the results of ophthalmic examination including presence and stage of retinopathy were recorded.

Serology

In every case, 2 mL of serum was stored at -20°C until analysis. Antibodies to Chlamydia were searched for by enzyme-linked immunosorbent assay (ELISA) (DIA Pro, Diagnostic Bioprobes Srl, Milano, Italy). The commercially available tests were performed according to the manufacturers' instructions. In brief, the diluted serum sample, controls and blanks were transferred into the wells of the ELISA plate. After 1-hour incubation at 37°C, the plate was washed three times. The conjugate (goat-antihuman-IgG antiserum HRPO conjugated) was then added to each well and the plate was incubated again for 1 hour. After three washing steps, substrate was added for a 30-minute period of incubation; the reaction was stopped by the addition of stop solution. The reaction was read by a microtiter plate reader (AT 400, SLT Laboratory Instruments, Salzburg, Austria) at 405nm (reference 490nm) for recombinant LPS ELISA, and at 450nm for C. pneumonia-specific ELISA. The cut-off values and values for positive and negative results were calculated according to manufacturers' instructions. All assays were performed and interpreted by a single investigator who was unaware of case or control status. An IgG level of more than 5 U/mL and IgA level of more than 0.357 U/mL was considered positive.
Statistical analysis
After entering the data to a masked database, the analysis was performed by an unaware statistician, using the Statistical Package for Social Sciences (SPSS) version 11.5.0 (SPSS, Inc. Chicago, Illinois). The Student t-test, chi-square, and ANOVA were used for analysis of quantitative and qualitative data, respectively. A regression model analysis was used to omit possible confounding factors. The significance level was set at 0.05.

Results
During the study period, 39 patients fulfilled the inclusion criteria; however, 12 were excluded due to lack of consent or meeting the exclusion criteria. Clinical characteristics of the included patients are provided in table 1.

Table 1. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>NDR+NPDR</th>
<th>PDR</th>
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<tbody>
<tr>
<td>No</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Age (yrs)*</td>
<td>28.80±8.67 (18-46)</td>
<td>29.72±7.34 (18-42)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>4:11</td>
<td>4:8</td>
</tr>
<tr>
<td>DM duration (yrs)*</td>
<td>13.13±5.16 (4-24)</td>
<td>14.75±3.38 (10-22)</td>
</tr>
<tr>
<td>BCVA, OD*</td>
<td>0.84±0.18 (0.4-1.0)</td>
<td>0.17±0.32 (NLP-1.0)</td>
</tr>
<tr>
<td>BCVA, OS*</td>
<td>0.84±0.19 (0.5-1.0)</td>
<td>0.37±0.38 (NLP-1.0)</td>
</tr>
</tbody>
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* Mean±SD (Range)
OD: Oculus Dextre
OS: Oculus Sinistre
NLP: No light perception
NDR: No diabetic retinopathy
NPDR: Non-proliferative diabetic retinopathy
PDR: Proliferative diabetic retinopathy

Seven patients had no diabetic retinopathy, 8 were in NPDR stage and 12 had PDR. None of patients had obvious atherosclerotic vascular disease or known respiratory tract infection at the time of examination. There was no significant difference in age of the patients between two groups (Table 1). IgA titer was negative in all of sera (Table 2).

Table 2. Serology results

<table>
<thead>
<tr>
<th></th>
<th>NDR+NPDR</th>
<th>PDR</th>
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<tbody>
<tr>
<td>IgG* (U/mL)</td>
<td>18.48±24.64 (1.10-82.70)</td>
<td>24.98±27.44 (3.20-93.50)</td>
</tr>
<tr>
<td>IgA* (U/mL)</td>
<td>0.141±0.045 (0.095-0.261)</td>
<td>0.181±0.060 (0.101-0.287)</td>
</tr>
</tbody>
</table>

* Mean±SD (Range)
NDR: No diabetic retinopathy
NPDR: Non-proliferative diabetic retinopathy
PDR: Proliferative diabetic retinopathy

There was no significant difference between IgG and IgA titer and seropositivity on the basis of sex and duration of diabetes. Because of the small sample size, correlation between seropositivity or titer of IgG and IgA with the presence of significant macular edema (CSME) or ruberosis iridis could not be proven. However, IgG seropositivity was different between ETDRS-diabetic retinopathy groups ($\chi^2=15.396, df=6, P=0.017$) (Figure 1). Comparing to patients without PDR, rate of IgG seropositivity was higher among patients with PDR (60.0% vs. 91.7%; $\chi^2=3.48, df=1, P=0.062$) (Figure 2 and 3). Although IgG was negative in all patients, its titers were higher in patients with PDR than those without PDR, reaching the significance at $P<0.06$ level (0.181 vs. 0.141; $t=-1.99, df=25, P=0.057$) (Figure 4). We found no statistically significant serologic difference between DR and NDR groups; however, regarding the limited power of the study a correlation may be found with larger sample sizes.

We noticed different IgA titers between diabetics with obvious neurological complications (e.g. paresthesia) and those without subjective complaints (0.170 vs. 0.131, respectively; $P=0.097$). The IgG titer was lower in patients with a history of admission due to diabetic ketoacidosis (DKA) (12.06 vs. 25.29; $P= 0.085$). Also, a lower titer of IgG in those with shin spots was remarkable (10.15 vs. 25.30; $P=0.04$). IgA titer was higher in patients with systemic arterial hypertension, which is a possible confounding factor (0.21 vs. 0.14, $P=0.001$). Patients with cortical cataract had a higher titer of IgA (0.196 vs. 0.146; $P=0.031$).
**Figure 1.** IgG seropositivity between different stages of diabetic retinopathy.

* Florid diabetic retinopathy (FDR) was designated for the patients with a rapidly, uncontrollably progressive PDR, beyond the ETDRS classification.

**Figure 2.** IgG seropositivity between PDR and Non-PDR diabetics.
**Discussion**

Due to large social and economic burden of diabetic retinopathy, pathogenesis and risk factors of the disease have been under active research since several years ago. Involvement of endothelial cells of retinal vasculature and production of various endothelial growth factors are important steps in the disease process. Progression from NPDR to PDR seems to occur following endothelial damage and growth factors, such as vascular endothelial growth factor (VEGF), stimulate new vessel formation.\(^{21,22}\)

Endothelial damage may be a result of altered metabolic state in diabetic patients; however, sometimes there is a discrepancy between course of the disease and course of retinopathy. This rapid progression to the so-called “florid diabetic retinopathy” proposes involvement of other factors in the pathogenesis. As many other diseases, infections may play a role in this process. Various infectious agents, especially C. pneumonia, seem to be involved in vasculopathies.\(^{8-13}\) Furthermore, atherosclerosis is a risk factor for aggravation of diabetic retinopathy and C. pneumonia is a
suspected agent in the pathogenesis of atherosclerosis.\textsuperscript{4-6}

In a cohort study on 296 diabetics, Klein et al. showed that atherosclerotic cardiovascular diseases and increased plasma levels of LDL are independent risk factors for progression to PDR.\textsuperscript{6} Following the Koch's postulates for an infectious disease, Liu and Waters demonstrated a positive correlation between C. pneumonia and atherosclerosis and atherosclerotic cardiovascular diseases.\textsuperscript{13} So it is likely for a relation between C. pneumonia infection and PDR to exist.

C. pneumonia is an obligate intracellular parasite capable of producing chronic or persistent infection. It was first identified 20 years ago as a cause of acute upper and lower respiratory tract infections.\textsuperscript{23} It has a unique developmental cycle involving two morphological forms, the elementary body, and the reticulate body. The former is the infectious form and is adapted for extracellular survival, whereas the latter is the metabolically active and dividing form, adapted for intracellular multiplication. In addition, the organism may evolve into a persistent body, an intracellular, metabolically inactive, nonreplicating but viable form that allows it to maintain chronic infection and thereby a state of chronic inflammation that may contribute to the atherosclerotic process. C. pneumonia infection is ubiquitous, with an antibody prevalence of 50\% by age 20 and 70\% to 80\% at age 60 to 70. It accounts for 10\% of community-acquired pneumonia and 5\% of pharyngitis, bronchitis, and sinusitis.\textsuperscript{24}

A model for the role of C. pneumonia in pathogenesis of atherosclerosis is described as follow.\textsuperscript{13} The organism could gain access to the vascular endothelium during local infections of the respiratory tract. Infected leukocytes may disseminate the organism throughout the body, and activated macrophages carry the organism to the subendothelial layer of arteries. The organism acts as a stimulus for chronic inflammation, inducing the production of tissue factor, leukocyte adhesion molecules, and inflammatory cytokines including tumor necrosis factor (TNF)-\ Alpha, and interleukins 1, 2, and 6.\textsuperscript{25} TNF-\ Alpha increases expression of leukocyte adhesion molecules,\textsuperscript{26} cellular apoptosis, endothelial inflammation, and endovascular thrombosis and inhibits lipoprotein lipase, leading to altered lipid metabolism and accumulation of triglycerides in the bloodstream.\textsuperscript{27} Increased expression of adhesion molecules promotes leukocyte adherence, migration, and intimal inflammation. These inflammatory cytokines stimulate fibroblast and smooth muscle cell proliferation. It is noteworthy that the induced fibroblastic growth factor is a key mediator in progression of NPDR to PDR. It also promotes a hypercoagulable state by activating platelets, increasing hepatic synthesis of acute-phase proteins, and inducing tissue factor release from endothelium with activation of the coagulation cascade.\textsuperscript{13} Bacterial lipopolysaccharide antigen acts as a potent stimulus for macrophage activation.\textsuperscript{28} In addition, it binds to low-density lipoprotein (LDL), which can modify the lipoprotein, making it toxic or immunogenic to endothelial cells.\textsuperscript{29} Persistent in situ growth of the organism leads to a state of chronic inflammation, platelet activation, vasospasm, and thrombosis.

C. pneumonia may contribute to atherosclerosis and endothelial damage via antigenic mimicry and autoimmunity. Chlamydial heat shock protein 60 (cHsp60) has been isolated in macrophages within atheromatous tissue, colocalizing with its homolog, human heat shock protein (hsp).\textsuperscript{30} A potential mechanism of vascular wall weakening that has been suggested is that persistent infection with C. pneumonia through the expression of cHsp60 may trigger an autoimmune reaction against human hsp. cHsp60 has also been observed to activate macrophages stimulating TNF-\ Alpha and matrix metalloproteinase expression, which may contribute to vessel weakening and subsequent rupture.\textsuperscript{30}

Current study is a pilot study on 29 patients with insulin dependent diabetes mellitus, and different stages of diabetic retinopathy. Although not reaching the proposed significance level of 0.05, there was a difference of anti-chlamydial IgA and IgG titers between those with PDR and those without PDR (P<0.07). Larger studies are needed to prove the association.

Regarding the nature of a pilot study, current study has its own limitations in sample size and randomization. Also, evaluation of
chlamydial infection with polymerase chain reaction (PCR) seems to be a more accurate method than ELISA technique used in this study. What is the clinical implication of proving C. pneumonia as a new risk factor for PDR? As it is under active investigation for coronary artery disease, we should expect treatment and prophylactic benefits of anti-chlamydial antibiotic therapies in diabetic patients. Although it is still a matter of controversy, several studies showed a clinical benefit of antibiotics in prevention of further cardiovascular attacks in patients with acute coronary syndromes. However before recommendation of such a regimen for diabetic patients, further investigations as prospective cohort study of the correlation between C. pneumonia and PDR along with controlled, randomized clinical trials of anti-chlamydial antibiotic prophylaxis in diabetic retinopathy, are necessary.

Conclusion

In the current pilot study, we could demonstrate a difference in anti-chlamydial IgG and IgA positivity and titer between PDR and non-PDR diabetics (P<0.07). As a pathogen of endothelial cells, C. pneumonia deserves further investigation as a risk factor for diabetic retinopathy.

Acknowledgement

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Ethics Approval

The study was approved by the research and ethical board of Mashhad University of Medical Sciences (document no. 82086).

References


