Turkish Journal of Endocrinology and Metabolism, (2000) 1 : 9-13 **ORIGINAL ARTICLE**

Evaluation of Chlamydia Pneumonia in the Atheromatous Plaques of Type 2 Diabetic Patients

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The present stud y has been designed to determine the existence of Chlamydia pneumonia (C. pneumonia) within the atherosclerotic plaques in a prospectively studied consecutive series of patients requiring coronary or carotid revascularization and to compare the incidence of C. pneumonia infection in diabetic patients with that of nondiabetic patients.

In order to obtain atherosclerotic plaque specimens, a consecutive cohort of patients undergoing coronary artery bypass graft operation or carotid artery endarterectomy were enrolled for the study. A total of 30 atheroma plaque specimens (from 15 type 2 diabetic patients; 15 nondiabetic patients) were able to be obtained for the Chlamydial DNA polymerase chain reaction (PCR) analysis. Age, cigarette smoking, lipid profile including lipoprotein (a), C. pneumonia seropositivity, duration of diabetes, glycemic indices, serum fibrinogen levels and presence of hypertension were assessed as determinants of atherosclerotic risk factors.

The incidence of Chlamydial seropositivity of the diabetic patients (3/15) and that of the nondiabetic patients (4/15) were similar. C. pneumonia PCR revealed an absence of bacterial DNA in the atheroma plaques of the patients in both the diabetic and the nondiabetic subpopulations.

C. pneumonial DNA is absent in the atheromatous plaques of the diabetic and nondiabetic patients. Diabetic patients with atherosclerosis do not have an increased incidence of Chlamydial infections.

Key words: Chlamydia Pneumonia, diabetes mellitus, atherosclerosis, PCR

Introduction

Traditional risk factors for atherosclerosis namely, hypertension, high cholesterol, smoking, obesity, male gender, family history and diabetes mellitus, can not always explain the existence of atherosclerotic macrovascular diseases. Thus some other important risk factors which increase susceptibility to atherosclerosis need to be identified (1, 2). In searching for additional risk factors for macrovascular diseases, the potential role of infection in the development of atherosclerosis has recently been reevaluated (1, 2, 3). In the last decade, some common chronic infections (including cytomegalovirus, herpes viruses, Helicobacter pylori, and dental sepsis) have been found to play a role in the etiopathogenesis of atherosclerosis (3, 7). Recent studies have shown some evidence linking C. pneumonia with coronary heart disease (4, 8, 10). The mechanisms by which C. pneumonia, a well-known human respiratory pathogen, might influence cardiovascular risk are unknown. One hypothesis is that chronic infection

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might contribute to atherosclerosis indirectly by increasing the concentration of acute phase reactants and inflammatory mediators, such as sialic acid, fibrinogen, lipoproteins, C-reactive protein and certain cytokines or they may infect arteries directly and lead to endothelial damage (3, 11, 13).

The present study has been designed to determine the existence of C. pneumonia within the atherosclerotic plaques in a prospectively studied consecutive series of patients requiring coronary and carotid revascularization using polymerase chain reaction (PCR) technique, and to compare the incidence of C. pneumonia infection in diabetic patients with that of nondiabetic patients.

Materials and Method

In order to obtain atherosclerotic plaque specimens from a consecutive cohort of patients with angiographically documented atherosclerotic macrovascular disease, all patients undergoing vascular revascularization (including coronary artery bypass graft operation and carotid artery endarterectomy) at Hacettepe University Hospital between January 1997 and February 1999 who gave informed consent were enrolled for the study. A total of 30 atheroma plaque specimens (15 type 2 diabetic patients; 15 nondiabetic patients) were able to be obtained for the PCR analysis.

All patients with coronary artery disease were pretreated with aspirin, all being stopped at least 24 h before the surgery; beta-adrenergic blocking agents, calcium channel blockers, nitrates and dipyridamole. Diabetic patients were followed in a diabetes mellitus clinic and managed with hypoglycemic drugs and/or insulin in a standard way. The following variables were specifically recorded: General demographic details, years of diabetes duration, chronic complications of diabetes and smoking habits.

Blood specimens were collected after an overnight fasting of at least 10 hours, before the programmed surgery. Serum samples were assayed for cardiovascular risk factors including total serum cholesterol and triglyceride and lipoprotein levels, fibrinogen, fasting and postprandial plasma glucose, and Hb A1c levels. Serum samples were also assayed for IgG antibody to C. pneumonia using indirect immunofluorescence technique (Euroimmun, Germany). IgG titers with dilutions of 1/100 or greater were considered as seropositive.

Triglycerides and cholesterol were measured by commercial colorimetric assay (GPO-PAP and CHOP-PAP kit, respectively, Boehringer-Mannheim). HDL-cholesterol in plasma was determined by a precipitation-based method with phosphotungustic acid (14). LDL-cholesterol was calculated by Friedewald formula (15). Plasma glucose determinations were obtained from venous sampling after 12 hours of overnight fasting, by a glucose-oxidase method (Boehringer - Mannheim, Germany). Plasma fibrinogen determination was made by the clotting method of Clauss (STA compact analyser) (16). Lipoprotein (a) concentrations were measured using an ELISA method (Boehringer - Mannheim kit). The detection limit of this assay was 0.5 mg/dl. The intra- and interassay coefficients of variation were 5-12 % and 2-6 % respectively.

The diagnosis of diabetes mellitus was made in the clinical setting and according to the diagnostic criteria of the World Health Organisation Expert Committee on Diabetes Mellitus (17). BMI was calculated as weight (in kilograms) divided by height (in square meters). Blood pressure was calculated as the mean of the blood pressure in the right and left arms, measured while the patient was in a sitting position after a 5-minute rest. Hypertension was defined as systolic blood pressure of $\geq$ 140 and diastolic blood pressure of $\geq$ 90 mmHg and/or history of antihypertensive drug treatment. Information regarding smoking history was obtained through interviews. Retinopathy was documented by standard fundus examination in all the diabetic patients by the same experienced ophthalmologist. Clinical neuropathy was defined by an abnormal neurological examination, plus abnormal nerve conduction in at least two peripheral nerves with temperature controlled and the patient lying down.

Microalbuminuria was defined as urinary albumin excretion between 30-300 mg/day. Advanced nephropathy was defined by the presence of urinary albumin excretion of more than 300 mg/day and a creatinine clearance of less than 70 ml/minute.

Polymerase chain reaction

C. pneumonia DNA was analyzed by polymerase chain reaction (PCR) in the atheroma plaques obtained during coronary artery bypass surgery from the proximal aorta (n=18) and during carotid endarterectomy (n=12). Biopsy specimens were transferred to sterile microcentrifuge tubes containing TE (10 mM Tris, 1 mM-EDTA) buffer and transported to the clinical microbiology laboratory. Tissues were ground with a sterile grinder and resuspended in 100 µl digestion buffer containing 50mM Tris pH 8.5, 1mM EDTA, 5%-Tween 20 proteinase K 200 µg/ml, and incubated for one night at 37°C. The suspension was incubated for 10 min at 95°C and debris was removed by centrifugation at 12.500 rpm for 10 min. The supernatant was transferred to a clean tube and stored at -20°C until PCR analysis.

Primers specific to the 438 base pairs (bp) fragment of C. Pneumoniae DNA (HL-1 5' GTT GTT CAT GAA GGC CTA CT 3' and HR-1 5' TGC ATA ACC TAC GGT GTG TT 3') were used in amplification reactions. Amplifications were carried out in 50 µl volumes of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, 2.0 mM MgCl2, 50 mM of each dNTP, 20 pmol of each primer, 1 unit of taq polymerase (Promega) and 5 µl DNA sample. Forty cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1min and extension at 72°C for 90 sec were performed in an automated thermalcycler (MJR, PTC150). Reaction mixtures without DNA were used as negative controls. The presence of the 438 bp amplification product was analyzed by electrophoresis of 10 µl amplified mixture on 2% agarose gel. Gels were stained with ethidium bromide and photographed on UV-transilluminator.

Statistical Analysis

Differences between patient groups were assessed for statistical significance using the Student's t test (18) and the Chi-square (19). All results were expressed as means ± SD unless otherwise indicated. Statistical analysis was conducted using the SPSS for Windows software package, Release 6.0. Statistical significance was considered when a P value was 0.05 or below.

Results

The table gives the clinical and biochemical characteristics of the type 2 diabetic patients and the nondiabetic group.

The diabetic and nondiabetic groups were matched with respect to age, body mass index, history of smoking and serum lipid parameters. The mean duration of diabetes mellitus was 7.9 ± 2.1 years and the mean Hb A1c level was 7.2 ± 1.6 % for the diabetic group.

Table Descriptive characteristiscs of the study population.

	Nondiabetic Patients	Diabetic Patients	p
n	15	15	-
Age (years)	59.1±5.1	60.6±6.4	0.203
Sex (M/F)	9/6	8/7	0.713
Smoking	12/15	10/15	0.679
Hypertension prevalance	13/15	11/15	0.648
DM duration (years)	-	7.9± 2.1	-
Hb A1c (%)	-	7.2±1.5	-
Microalbuminuria (mg/day)	-	27.8 ± 20.4	-
LDL Cholesterol (mg/dl)	150.5± 129.7	144.8 ± 45.8	0.874
HDL Cholesterol (mg/dl)	44.9±11.6	47.5 ±12.1	0.553
Lipoprotein a (mg/dl)	359.4 ±137.6	357.8±132.4	0.974
Fibrinogen (mg/dl)	362.4±67.8	380.4±45.1	0.399
C.Pneumonia Ig G Seropositivity	4/15	3/15	0.666

There were no differences in the C.pneumonia titers between diabetic and nondiabetic patients (3/15 and 4/15 for the diabetic and the nondiabetic group respectively). C. pneumonia PCR revealed the absence of bacterial DNA in the atheroma plaques of the patients in both the diabetic and the nondiabetic patients.

Discussion

Atherosclerosis is pathologically similar to chronic inflammatory response. Injury of the endothelium, subendothelial migration and accumulation of macrophages, proliferation of smooth muscle cells and local production of adhesion molecules and growth factors are considered to be the key factors in the pathogenesis of atherosclerosis (11, 20). Diabetes mellitus is an important cause of premature death due to accelerated atherosclerosis, clinically evident as coronary, cerebrovascular and peripheral vascular disease. Despite the fact that infectious diseases are more common in diabetic than in nondiabetic patients, available data is not enough about the prevalence of Chlamydial infections and their role in the development of cardiovascular disease in diabetic subjects are not sufficient (4, 21). Asymptomatic infection with C. Pneumonia is worldwide and surveys in various parts of the world have demonstrated that preexisting antibodies to C. pneumonia occur in 40-60% of the adult populations (22, 23). In the present study the incidence of C. pneumonia seropositivity was found to be similar in the diabetic and the nondiabetic subpopulations. Hence, in this small population of atherosclerotic patients, the diabetic subpopulation was not found to have increased susceptibility to Chlamydial infections when compared with the nondiabetic subpopulation.

In this cross-sectional study, none of the atheroma plaques were infected with the bacteria as demonstrated by the negative PCR amplification results. Because the sensitivity and specificity of this nonculture test, PCR, are not known, the results of the study with a relatively small number of patients may represent false negative results. The role of chronic infections including Chlamydial infections in atherosclerosis is not fully defined. As far as the negative Chlamydial PCR amplification results are concerned, an indirect contribution to endothelial damage via acute phase reactants and inflammatory mediators, such as sialic acid, fibrinogen, lipoproteins, C-reactive protein might be more relevant than their direct toxicity to the macrovasculature through infection of the endothelial cells (3, 11, 12, 24, 29).

A causal relation between C. Pneumonia and atherosclerotic plaque formation will have to be shown by further investigations. The results of this study suggest that diabetic patients with atherosclerosis do not have an increased incidence of C. Pneumonia infection when compared with the nondiabetic population and probably C. pneumonia does not have a direct toxic effect on the vascular endothelium. Although no firm conclusions can be drawn about the association between C. pneumonia and atherosclerosis, an indirect causal effect of Chlamydial infections on atherosclerosis can only been seen as a hypothesis (generating) and needs further studies with larger groups of patients. The clarification of the influence of any factor on the development of the atherosclerotic diseases could contribute to their control and prevention.

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