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The isolation of chlamydia pneumoniae in atherosclerosis patients in Iran by PCR method

Research Article

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Summary

Cardiovascular disease (CAD) is the leading cause of death in developed countries. The cause is multifactorial. A substantial proportion of patients with CAD do not have traditional risk factors. Infectious diseases may play a role in these cases, or they may intensify the effect of the risk factors. The association of CAD and Chlamydia pneumoniae infection is firmly established, but causality is yet to be proven. We investigated their presence in carotid atherosclerotic plaques. 102 plaque atherosclerotic in dead patients were available for examination in Tehran, Iran. The highly sensitive polymerase chain reaction method was employed with primers specific for this agent. The presence of Chlamydia DNA was detected in 23 (22%) out of 102 examined samples. The presence of Chlamydia DNA in these patients supports the hypothesis that this agent has an association with atherosclerosis.

I. Introduction

Only half of coronary artery disease, and half of carotid plaque measured by ultrasound, can be explained by the usual risk factors: age, sex, hypertension, hyperlipidemia, smoking, and diabetes. It is likely that much of unexplained atherosclerosis is genetic: a Swedish twin study showed that myocardial infarction heritable this suggests that few environmental factors remain to be discolved that would make a major contribution to atherosclerosis.Recently, the notion that infection may be important in atherosclerosis has been of interest.(Spence and Norris, 2003).

Recently, a potential link between infectious agents and atherosclerosis has been suggested. Data obtained from several seroepidemological studies has given rise to the hypothesis that an infection can initiate or maintain the atherosclerotic process (Farsak et al, 2000). *Chlamydia pneumoniae* is a common cause of a usually mild, community acquired pneumonia. This organism, however, can spread from the respiratory tract into other parts of the body and has been detected in up to 70% of atheromatous lesions in blood vessels. Although the exact mechanism of the contribution of *C. pneumoniae* to the pathogenesis of atherosclerosis remains unknown (Cagli et al, 2003).

Similarly, there is increasing evidence that *Chlamydia pneumoniae*, a common respiratory tract pathogen, may play a role in atherosclerosis. *C. pneumoniae* has been associated with coronary and carotid artery disease in seroprevalence epidemiological studies, and in one prospective cohort study *C. pneumoniae* elementary bodies have been detected in the atherosclerotic plaques and fatty streaks of the aorta and coronary arteries of autopsy cases. From atherectomy specimens of coronary arteries, and from endarterectomy specimens of carotid arteries (Fong, 2000).

In the present study, the presence of *C. pneumoniae* was investigated by PCR in arterial plaque, as was the correlation between the clinical status and DNA positivity of these bacteria.

II. Materials and methods

A. Study design

The research in this study has been done by descriptive methods. Samples were obtained in 2002 from 102 dead cases with infarction due to Atherosclerosis. Basic demographic data

and clinical information such sex, smoking, familiar heart problem history coronary artery diseases, diabetes, blood pressures and level of blood cholesterol (LDL & HDL) in sera were extracted either from the files

B. DNA extraction for chlamydia

DNA from 50 µl of homogenized tissues was isolated by proteinase K digestion (100 µg/ml for 1 to 2 h at 65°C) followed by phenol-chloroform extraction and ethanol precipitation.The DNA was then resuspended in 50 µl of Tris-EDTA buffer. For PCR, 10 µl of DNA solution, was added per 50 µl of reaction mixture (Skowronski et al, 1993).

C. PCR amplification

PCR targeting the 16S rRNA gene and a nested PCR targeting the ompA gene were performed to detect *C p*neumoniae DNA. All amplification reactions were done in a volume of 50 µl containing 200 µM of four deoxynucleoside triphosphates. PCR primers tested were CPN90 5' GGT CTC AAC CCC ATC CGT GTC GG 3', CPN91 5' TGC GGA AAG CTG TAT TTC TAC AGT T 3', CP1 5' TTA CAA GCC TTG CCT GTA GG 3', CP2 5' GCG ATC CCA AAT GTT TAA GGC 3' (Cagli et al, 2003).

Briefly PCR was performed using CPN90-CPN91 primer pair with a 0.25 µM concentration of each primer, 2.5 mM MgCl2 and 20 µl of the extracted DNA. Cycling protocol was 75 seconds at 95°C, followed by 60 cycles of denaturation at 94°C for 45 seconds, annealing beginning at 64°C and ending at 52°C for 45 seconds, and extension at 72°C for one minute. The annealing temperature was lowered 10°C every four cycles until 52°C and this temperature was kept until the end of the cycling process.

CP1-CP2 primers with nested pair CPC-CPD were used for the ompA nested PCR. The first round of amplification used 1.5 mM $MgCl_2$, 0.4 µM of each primer and 20 µl of the extracted DNA. Cycling consisted of nine minutes at 95°C for Taq polymerase activation, 20 cycles of one minute at 94°C, one minutes at 65°C (temperature was decreased 0.5°C for each cycle) and one minute at 72°C plus an additional 20 cycles of one minute at 94°C, one minute at 55°C and one minute at 72°C. The PCR products amplified by the outer primer pair were diluted 1:5 and 5 µl was added to a new PCR mixture containing 1 µM of each primer and 3 mM of $MgCl_s$. Cycling protocol entailed nine minutes at 95°C for Taq DNA polymerase activation, 30 cycles of one minute at 94°C, one minute at 50°C and one minute at 72°C.

III. Results

In Total, 102 patients (20 to 79 years old) **Figure 2** from 75 males and 27 females had been identified and died from atherosclerosis. Of these patients Chlamydia DNA was detected in 23(22%) (**Figure 1**).

100(98%) of 102 atherosclerosis patients had the primary obstruction and the rest (2%), the secondary obstruction. on the other hand majority of obstruction (91%) were been displayed in left anterior decending artery (LAD).

Among of the Chlamydia DNA positive patients (**Picture 1 and 2**), some risk factors (sex, hyperlipidemia, blood pressure, diabetes, smoking and family history of premature cardiovascular disease) were established. Out of 102 patients with atherosclerosis, 20 (36.30%) increasing of LDL, 18 (47.30%) decreasing of HDL, 6 (24%) increasing of blood pressure, 7 (38.80%) diabetes, 12 (24%) smoking and 8 (29.90%) family history of premature cardiovascular disease identified with DNA C.pneumoniae (**Figure 1**).

IV. Discussion

Human atherogenesis appears to be of multifactorial etiology, and no single entity can fully explain the pathogenesis. There is little doubt that risk factors such as genetic predisposition, hypercholesterolemia, hypertension, smoking, and diabetes mellitus are major predisposing conditions for atherosclerosis. There is substantial evidence, albeit circumstantial, those infectious agents are associated with atherosclerosis, but their exact role in the pathogenesis of atherosclerosis is unknown. The most compelling evidence to date is the presence of infectious agents in the arterial wall, particularly in diseased vessels or within atherosclerotic plaques (Chiu et al, 1997).

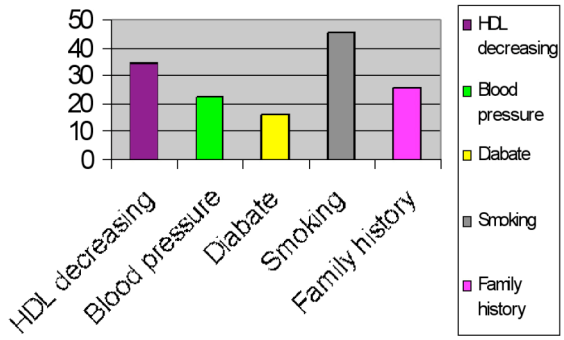

Figure 1. The frequency of risk factors in Atherosclerosis patients with Chlamydia Pneumoniae (1-DNA Chlamydia Positive and 2 –DNA Chlamydia negative).

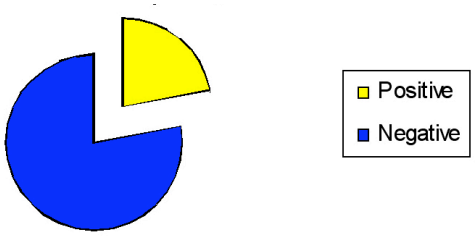

Figure 2. The frequency of Chlamydia positive in Atherosclerosis patients.

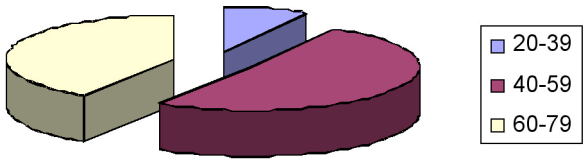

Figure 3. The frequency of age in Atherosclerosis patients.

C. pneumoniae, an obligate intracellular gram-negative bacterium, has been associated with atherosclerotic cardiovascular disease both by seroepidemiological studies, indicating a significantly higher prevalence of circulating *C.* pneumoniae antibody

or immune complexes among persons with clinical or radiographic evidence of atherosclerotic disease. C *pneumonia*e has now been detected in atherosclerotic plaques in several different arterial sites (coronary arteries, aorta, and carotid arteries) and in early lesions (fatty streaks) and through the use of various independent techniques. The organism has been detected by electron microscopy, immunocytochemisty, direct immunofluorescence, and the PCR in coronary artery and carotid artery plaque specimens (Shor et al, 1992; Kuo et al, 1993a, b; Chiu et al, 1997; Farsak et al, 2000).

Some study was about comparison trial of DNA extraction methods and PCR assay for detection of *Chlamydia pneumoni*ae in endarterectomy specimens. There was no consistent pattern of positive results among the various laboratories, and there was no correlation between the detection rates and the sensitivity of the assay used (Apfalter et al 2001).

Bartels et al, even found that occluded aorta-coronary venous grafts harbour *C pneumoniae* (Bartels et al, 2000).

Using PCR and immunohistochemistry, C pneumoniae was detected in arterial biopsies from femoral, popliteal, and coronary arteries, as well as in the aorta, indicating that the organism is widespread in atherosclerosis of the vascular system (Kuo et al, 1993a; Davidson et al, 1998). Some studies found Between individuals, the percentage of arteries with immunoreactivity to *C pneumoniae* was associated with the average area stenosis throughout the arterial system. Their conclusion displayed *C pneumoniae* was mostly observed at locations that are related to clinically relevant features. Within the individual, the distribution of C *pneumoniae* is associated with the distribution of atherosclerosis (Vinik et al,2001).

On the other hand, Andreasen et al, could not detect C pneumoniae in calcific or degenerative atherosclerotic aortic heart valve disease and Nystromrosander et al did detect C pneumoniae in aortic valves using electron microscopy (Nystrom-Rosander et al, 1997; Andreasen et al, 1998).

Furthermore, it is unclear whether C pneumoniae initiates the process of atherosclerosis, facilitates progression of existing plaques, or merely colonises the lesions. Some study Shown that the adventitia of atherosclerotic coronary arteries frequently contains C. *pneumoniae* that seems to be located within macrophages. These results might indicate a possible route for infected circulating macrophages to home into atherosclerotic lesions in the artery via vasa vasorum (Vink et al, 2001a).

Another study was to determine the presence of C. *pneumoniae* in coronary artery plaques, carotid artery plaques and old vein grafts that were harvested at the time of surgery. But it failed to find C. *pneumoniae* in any of the vascular tissue. So was concluded that a large cooperative study involving surgical specimen analysis is needed to assess the role of C. *pneumoniae* in the etiology of atherosclerosis (Johnson et al,2001).

In our study C. *pnemoniae* was detected in 22 (23%) out of 102 tissue plaques from dead atherosclerosis patients. Also, there were so many risk factors in that patients. Therefore, In a condition with so many risk factors and genetic influences it seems unlikely that infection will be the only or main cause of atherosclerosis and events. The role of these newly emerging risk factors and their relationship with traditional risk factors such as hypertension or lipids, remains unexplored. The uncertainty of their role and the types of infection or types of patients that should be treated must be explored in properly conducted, prospective studies. However, the findings to date are intriguing, and the hope that anti-infective therapy may reduce the burden of stroke is worth pursuing.

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