
PATHOHISTOLOGICAL CHANGES IN DIFFUSE CORONARY ATHEROSCLEROSIS AND CHRONIC INFECTION CAUSED BY CHLAMYDIA PNEUMONIAE

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ABSTRACT

BACKGROUND AND PURPOSE:

To investigate the histopathologic characteristics of atherosclerotic lesions in diffuse coronary artery disease and to evaluate the possible inflammatory role of chronic infection with Chlamydia pneumoniae (CP).

MATERIALS AND METHODS:

For 10 patients (males, mean age 61 years) who were surgically treated for grave diffuse coronary artery disease, histomorphological analyses of endarterectomized segments of the coronary arteries were performed. Serological analyses for the detection of CP antibodies in peripheral blood were done, preoperatively.

RESULTS AND CONCLUSIONS:

Diffuse and concentric atherosclerotic changes from VI to VIII stage according to the Stary classification were found. Immunohistochemical methods revealed infiltrates of T-lymphocytes (80% of cases), B-lymphocytes (40% of cases) and macrophages (80%). Using the nuclear marker for proliferation activity MIB-1, single MIB-1 positive cells were found in 40% of cases. Features of arteriogenesis and vasculitis of newly formed arterioles (as well as thickening of the wall of newly formed arterioles) were found in the vessel wall of 8 patients, 7 of them had chronic infection with CP (preoperative micro-immunofluorescent test results: 1:32 < IgG ≤ 1:512 and IgA ≥ 32), one had passed CP infection (1:32 ≤ IgG < 1:512, IgA negative). These features were absent in 2 patients, both recovered from CP infection and had not the chronic CP infection at the time of surgery. DNA of Chlamydia pneumoniae was detected using the polymerase chain reaction (PCR) method in the vessel wall of 3 patients who were chosen randomly for this method. This study suggests an inflammatory and proatherogenic role of CP in a high grade atherosclerotic coronary artery wall in diffuse coronary artery disease.

INTRODUCTION

The modern view on the aetiopathogenesis of atherosclerosis includes the inflammatory process on the vessel wall. This inflammation might at least partly be caused by certain infectious agents, among them Chlamydia pneumoniae is a visible candidate (1, 2). Pathogenetic mechanisms of possible Chlamydial involvement in the progression of atherosclerosis have been already discussed (3). Seroepidemiological, laboratory and pathological studies (4-7) revealed CP as »being there« in atherosclerosis, however, whether CP is an initiator, promoter or an innocent bystander in the atherosclerotic process remains unproven (8).

Diffuse coronary atherosclerosis is a special entity of coronary atherosclerosis where long segments of coronary arteries are diffusely atherosclerotically damaged (9). During the surgical treatment with the classic by-pass technique, many times it is necessary to make an endarterectomy of the diffusely involved artery segment. This is the procedure where the surgeon with a special knife has to cut off the damaged intima of the coronary artery wall (10). Endarterectomized segments offers the unique opportunity for studying the atherosclerotic vessel wall.

The aim of our research was to study pathohistological changes in the endarterectomized segments of the coronary artery wall in patients with serologically proven chronic CP infection where we were expecting to find distinct pathomorphological features regarding the patients without chronic CP infection.

MATERIALS AND METHODS

We histologically analysed the endarterectomized segments of the coronary arteries for 10 patients who were surgically treated because of diffuse coronary artery disease. All our patients were males, their mean age was 61±3 years. Eight of them suffered myocardial infarction before the operation, and angina pectoris was present for 3-24 months before the operation. All patients had arterial hypertension, their mean blood pressure was 145/90 mm Hg (receiving therapy!). Also, all were obese,

their mean BMI was 30.0±4.4 kg/m². Nine patients were hyperlipidemic and had been receiving therapy with statin, 4 patients had diabetes. 90% of our patients were smokers, preoperatively.

Immediately after the operation, the tissue samples of the endarterectomy were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Four mm sections were cut, deparaffinized, and stained with hematoxylin-eosin (HE), Masson-trichrome and Weigert. For the immunohistochemical analyses the sections were heated in a microwave oven (15 minutes for CD3, CD79 α , CD68, and 25 minutes for the proliferation marker Ki-67). For the detection of the proliferation marker Ki-67, the MIB-1 monoclonal antibody was used. The sections were washed with a phosphate-buffered saline solution (PBS). Then, the primary antibodies were applied: CD3 monoclonal antibody, incubation overnight in a wet chamber at 4°C (DAKO, Glostrup, Denmark; dilution 1:40); CD79 α monoclonal antibody, incubation for 30 minutes at room temperature (DAKO, Glostrup, Denmark; dilution 1:80); CD68 monoclonal antibody, incubation for 30 minutes at room temperature (DAKO, Glostrup, Denmark; dilution 1:40); MIB-1 monoclonal antibody, incubation overnight in a wet chamber at 4°C (DIANOVA, Hamburg, Germany; dilution 1:100). After washing in PBS, streptavidin-biotin complex/horseradish peroxidase was applied for 30 minutes at room temperature. Positive controls for CD3, CD79 α , CD68, and MIB-1 tonsils were used.

In the step serial sections of endarterectomized segments, the severity of atherosclerosis was graded according to the Stary classification (11, 9). Cell infiltration, proliferation activity of cells and tissue, capillarogenesis and arteriogenesis in the endarterectomized segments were investigated.

Preoperatively, a venous blood sample was taken for the determination of the antibody levels IgG, IgM and IgA to CP, at least 3 times in each patient over a period of 6 months to prove a stable titer. Serological studies were performed in the Institute of Microbiology of the Medical Faculty, University of Ljubljana by the microimmunofluorescence method (MIF), utilising *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Chlamydia trachomatis* elementary bodies (MRL Diagnostics, USA) as antigens to detect specific IgG, IgM and IgA antibodies. Serological evidence of CPn infection was based on the criteria published by Grayston et al. (12). A fourfold rise in IgG/IgA titer in paired sera or an IgM titer of $\geq 1:20$ in any serum were considered as presumptive evidence of **acute or recent infection** with CP. Titers of IgG $\geq 1:32$ and $< 1:512$ were presumed to be due to **past infection** with Cp. Titers with stable IgG and IgA titers $\geq 1:32$ were presumed to be **chronic infection** with CP. A **negative result** was defined as an IgG/IgA titer $< 1:32$ and IgM titer $< 1:20$. The sera tested for IgM or IgA antibodies were pre-treated to remove possible free and complexed

IgG antibodies, following the manufacturer's instructions (MRL Diagnostics, USA).

The study was approved by the National Ethics Committee and an informed consent was obtained by each patient

RESULTS

Seven of our patients had chronic infection with CP ($1:32 < \text{IgG} \leq 1:512$ and $\text{IgA} \geq 1:32$) and 3 patients had past infection with CP ($1:32 \leq \text{IgG} < 1:512$ and IgA negative). These 3 patients served us as a small control group. In all 10 patients infection with other types of *Chlamydia* (*trachomatis*, *psittaci*) was excluded.

Histopathologic examination of step-serial hematoxylin-eosin sections of endarterectomized segments revealed that the arterial intima was diffusely and concentrically atherosclerotically involved, with narrowed lumen and proliferated intima (Picture 1), sometimes with cholesterol crystals in lipid core diffuse atheromas, but predominantly with proliferative fibrous tissue, somewhere calcified.

The greatest stage of atherosclerosis (according to Stary classification) – type VIII lesions – were found in 6 patients, type VII lesions were found in 2 patients and type VI to VII lesions in 2 patients. Macrophage infiltrations were found in 80% of cases, T-lymphocytes in 80%, B-lymphocytes and plasma cells in 40%. Single MIB-1 positive cells were found in 40% of cases, either in the area of mononuclear infiltrates or outside the area of mononuclear infiltrates, where these cells were confined predominantly to the area of fibrointimal hyperplasia (see Picture 2). Single MIB-1 positive cells were also found among smooth muscle cells in the vessel wall of arterioles.

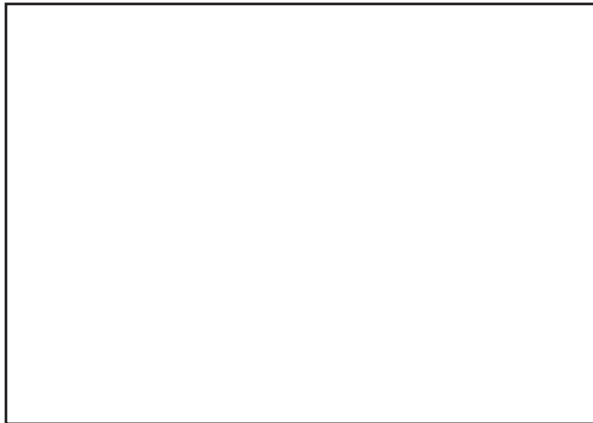
We found neoangiogenesis in the inner part of atherosclerotically changed intima in 80% of cases. In 70% of cases it was capillarogenesis, in 60% we found newly formed arterioles and small arteries. Both forms, capillarogenesis and arteriogenesis, were found in 50% of cases. No angiogenesis was found in 20% of cases; these were 2 patients without chronic CP infection.

Most newly formed arterioles and small arteries had thickened and hyalinized vessel walls. In 60% of cases, arterioles showed features of vasculitis – in their vessel walls and perivascularly the infiltration of polymorphonuclear cells (neutrophils) was found (Picture 3).

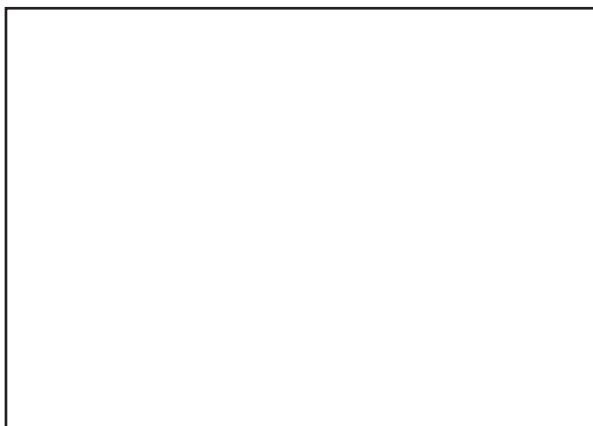
We had the opportunity to test 3 (2 with chronic and 1 with past CP infection) out of 10 patients for the presence of CP DNA in the vessel wall with the PCR method. In all 3 cases we obtained positive results – CP DNA was found.



Picture 1. Cross section through the endarterectomized segment of the coronary artery: the intima is diffusely and concentrically atherosclerotically involved. The diffuse mononuclear infiltrate is visible as well as neoangiogenesis in the outer parts of the intima. (x25)



Picture 2. Single MIB-1 positive cells (arrow) in the connective tissue of the atherosclerotic plaque and a single MIB-1 positive smooth muscle cell in the vessel wall of the arteriole/small artery. (x 320)



Picture 3. Vasculitis of newly formed arteriole. In the perivascular infiltrates, neutrophils are found. (x160)

DISCUSSION

The patients with chronic CP infection and the patients without chronic CP infection did not differ in the stage of atherosclerosis or the extent and severity of mononuclear infiltration (regarding macrophages and T-lymphocytes), but B-lymphocytes and plasma cells were more commonly found in the atherosclerotically changed coronary artery wall of patients with chronic CP infection. However, arteriogenesis was the unique morphologic feature in chronic CP infection as well as the vasculitis of newly formed arterioles and small arteries. Indeed, in one patient with past CP infection these signs were also present; it is possible that seronegativity was achieved recently, since the CP-DNA was still present in his vessel wall. In the last decade it is well known that classical risk factors such as hyperlipidaemia, hypertension, diabetes, obesity and smoking do not account for all the incidence of atherosclerosis and cardiovascular disease. Other risk factors have been investigated and infectious agents and their possible role in the atherosclerotic process became apparent (13). The connection between CP and coronary artery disease was first mentioned in 1988 by a Finnish research group (14) and was supported by many references afterwards (15, 16). However, although possible pathogenetic mechanisms are already known (3), it is still unknown whether CP plays a causal, contributory or bystander role.

Our study contributes to the evidence that CP might play the contributory role in the genesis of atherosclerosis. A special histomorphologic picture has been found in endarterectomized segments of atherosclerotically damaged coronary arteries. These changes speak for the presence of chronic inflammation and immune reaction (B-lymphocytes, plasma cells, neoangiogenesis). Finding newly formed arterioles and small arteries is highly suggestive of the complex pattern of pathogenetic process. Indeed, ischemia in atherosclerotic tissue induces capillarogenesis (17) and this is the expected finding in atherosclerotic tissue. However, ischemia is an unlikely stimulus for arteriogenesis/arteriogenesis, since vascular endothelial growth factor (VEGF), the only growth factor with a clear connection to hypoxia, is not a mitogen for smooth muscle cells that form arteriolar wall (18). Other cytokines (FGF, IGF-1, TGF-beta), released during inflammation from monocytes and lymphocytes, trigger the proliferation of smooth muscle cells in arteriogenesis and arteriogenesis. We believe that growth factors released from monocytes and lymphocytes at inflammation caused by CP, influence the formation and growth of arterioles and small arteries. Interestingly, it was quite common to find MIB-1 positive cells (in 40 % of all cases), mostly in endothelial cells, fibroblasts and also in smooth muscle cells of arterioles, indicating the extensive proliferating activity of these components of the atherosclerotic artery wall.

Moreover, finding vasculitis of the newly formed vessels (in 60% of arterioles and small arteries, vascular and perivascular infiltration with neutrophils was found) shows that the damaging process is continuing, leading to further damage and the early atherosclerosis of newly formed vessels. It is important to emphasise that all these special pathohistological signs were absent in two patients without chronic CP infection (they had only past CP infection). However, in the group of 3 patients with past CP infection, there was one with arteriogenesis and vasculitis; probably, seronegativity was achieved recently, moreover since CP-DNA was still present in his

vessel wall.

Finding CP-DNA with the PCR method in all 3 patients who were randomly selected for this method is another proof for CP being present in the artery wall not to rely only on serological criteria for chronic infection.

In conclusion, our study speaks for the contributory and continuous role of CP in atherosclerosis in diffuse CAD. Inflammatory and immune responses in the artery wall are triggered in the chronic infection with CP and a special histomorphologic picture in the atherosclerotic tissue of endarterectomized segments of coronary arteries was found.

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