In vitro Degradation of Aortic Elastin by Chlamydia pneumoniae

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Objectives: to investigate whether Chlamydia pneumoniae (C. pneumoniae) may increase elastin degradation in the aortic wall.

Materials and Methods: eighteen full thickness aortic wall samples from non-aneurysmal infrarenal abdominal aortas were collected from autopsies. Two adjacent and equally large pieces were cut out of each aortic sample. From each sample, one piece was incubated in a HEp-2 cell culture infected with C. pneumoniae and the other piece was incubated in an uninfected HEp-2 cell culture. The incubation time was one week at 35°C. The concentration of elastin-derived peptides (EDP) (ng/ml) in the medium of each cell culture was measured in duplicate. For each paired sample, delta-EDP (EDP in HEp-2 cell culture infected with C. pneumoniae – EDP in uninfected HEp-2 cell culture) was calculated.

Result: there was a significantly increased degradation of aortic elastin, estimated by EDP concentrations in cell culture conditioned medium, when aortic wall samples were incubated in C. pneumoniae cultures compared with uninfected cultures (p=0.025, Wilcoxon signed ranks test).

Conclusion: these results indicate that there is a relationship between the presence of C. pneumoniae and increased elastin degradation in the aortic wall in vitro. This suggests C. pneumoniae in the aortic wall directly or indirectly leads to the degradation of aortic elastin.

Key Words: Abdominal aortic aneurysms; Aorta; Chlamydia pneumoniae; Elastin degradation.

Introduction

The obligate intracellular bacterium Chlamydia pneumoniae (C. pneumoniae) has recently been demonstrated in the wall of abdominal aortic aneurysms (AAA), and viable C. pneumoniae has been cultured from AAA specimens. Whether this intracellular bacterium is an "innocent bystander" or actively involved in the development of AAA is unknown.

C. pneumoniae is linked to atherosclerosis and AAA may be a late manifestation of atherosclerosis in the distal aorta. The inflammatory process in atherosclerosis and in AAA disease in particular suggests that some infectious agent may be involved in its pathogenesis. C. pneumoniae has been identified in the infiltrating macrophages, and it has been shown that C. pneumoniae-specific T lymphocytes are present among in vivo activated cells from AAA wall specimens. This may suggest that C. pneumoniae is involved in the development and progression of AAA disease. Proteinases derived from infiltrating inflammatory cells in the AAA wall cause increased elastolytic activity and degradation of elastin, which is the principal load-bearing protein in the aortic wall. Reduced elastin concentration is a characteristic finding in AAA walls and a contributing cause of dilatation. The identification of an association between elastin degradation in the aortic wall and C. pneumoniae would strengthen the hypothesis that C. pneumoniae is actively involved in AAA disease.

The aim of this study was to investigate whether C. pneumoniae may increase elastin degradation in the aortic wall.

Materials

Eighteen full thickness aortic wall samples from non-aneurysmal infrarenal abdominal aortas were collected from autopsies performed at the Department of Pathology, Umeå University Hospital. The samples were collected within 48h after the time of death and immediately frozen at −70°C. The Scientific Ethical Committee of Umeå University Hospital approved the
study and the aortic samples were collected according to the Law of Transplantation (SFS 1975: 190).

**Methods**

**Incubation of aortic wall samples**

Forty cultures with a monolayer of HEp-2 cells were prepared on well plates. Twenty of the cultures were inoculated with a *C. pneumoniae*-strain (T45). Two adjacent pieces of 500 mg were cut out of each of the 18 aortic samples. One piece was applied to a well inoculated with *C. pneumoniae* and the other piece was applied to a well without *C. pneumoniae*. Four wells, of which two were inoculated with *C. pneumoniae*, were left as controls without aortic tissue. After centrifugation for 1 h at 2500 × g, the plates were incubated at 35°C in 5% CO₂ in an RPMI-1640 medium (Sigma, St. Louis, U.S.A.) supplemented with 10% fetal calf serum, 0.5% glucose, 2 μg/ml of cycloheximide, 8 μg/ml of garamycin (Schering-Plough), 1 μg/ml of fungi-zone (Bristol-Meyers Squibb) and 25 μg/ml of vancocin (Lilly). After 7 days the supernatant was collected and analysed.

**Preparation of antibodies**

To get soluble alpha-elastin, insoluble elastin from bovine neck ligaments was treated with 0.25 M oxalic acid at 90°C. The alpha-elastin was dissolved in phosphate buffer saline (PBS) to a concentration of 2 mg/ml, followed by emulsion in an equal volume of Freund’s complete adjuvant. New Zealand White rabbits were immunised by multiple subcutaneous injections using complete Freund’s adjuvant. All of the rabbits were boostered fortnightly over a period of 3 months. The rabbits were bled by ear venous puncture 10 days after the last injection, and serum was collected by centrifugation. Immunoglobulins were purified using protein-A affinity chromatography and dialysed against PBS and stored in a concentration of 2 mg/ml at 4°C.

**Competitive ELISA**

The wells of the microtiter plate were coated with 100 μl of α-elastin (1.25 μg/ml) in 0.1 M sodium carbonate, pH 9 and incubated for 12 h at 4°C. For generation of a standard curve, anti-α-elastin antibodies (2 μg/ml) in PBS containing 0.05% tween 20 and 7% bovine serum albumin (PBST-7% BSA) were pre-incubated with a variable concentration of α-elastin peptides (0–120 ng/ml) in PBST-7% BSA for 36 h at 4°C. Simultaneously, cell culture medium was mixed with anti-α-elastin antibodies (2 μg/ml) in PBST-7% BSA and incubated for 36 h at 4°C. 200 μl of these reactions were transferred to the α-elastin coated microtiter plate in triplicate. Before adding the reactions the plate was washed 4 times with 400 μl of PBST in an automated plate washer (Labsystem Multiwash). The plate was incubated for 1 h at room temperature and washed with PBST. 200 μl of HRP-labeled donkey-anti-rabbit IgG antibody (1:4000, Amersham Pharmacia Biotech, NA 934) were added and incubated for 1 h at room temperature and washed with PBST. Thereafter 200 μl of phosphate-citrate buffer, pH 5.0, containing 2 mg/ml of o-phenylendiamine dihydrochloride (Sigma, P7288) and 0.012% H₂O₂ were added and the plates were incubated in the dark for 20 min at room temperature. Reactions were stopped by adding 50 μl of 3 N HCl. Absorbency was read at 492 nm using an automated plate reader (Labsystem, Multiscan MS). The concentrations of EDP were expressed as ng/ml.

The investigator measuring EDP concentrations was not aware of which media came from *C. pneumoniae* infected or uninfected cultures, respectively.

**Statistics**

The Statistical Package for the Social Sciences (SPSS 10.0) was used. Wilcoxon signed-ranks test was used to test for differences between the paired samples. Results were given as the median and range. Statistical significance was set at 95% (p<0.05).

**Results**

The median age of the patients from which the aortic samples were collected was 80 (range 53–90) years. Six of the aortic samples were collected from females. The aortic sample from cases number 2, 3, 5, 6, 11, 12, 14 and 15 (Fig. 1) showed atherosclerotic calcifications. There were no signs of atherosclerotic ulceration, thrombosis or dissection in any of the patients’ distal aorta.

After incubation of the aortic wall pieces, there were significantly higher concentrations of EDP in media from HEp-2 cell cultures inoculated with *C. pneumoniae* (2.64 ng/ml, range 0.41–9.15) than in HEp-2 cell cultures without *C. pneumoniae* (1.42 ng/ml, range 0.08–
between cell cultures, such as the degree of apoptosis or the concentration of \(C. pneumoniae\) may be raised. Additionally, variations, possibly genetic in origin, in enzymes or connective tissue types between the aortic samples, may influence the result. Defects in the cross-linking of mature elastin may increase the susceptibility to degradation.

However, the relationship between \(C. pneumoniae\) and elastin degradation in this in vitro experiment may not be extended to apply to in vivo conditions. We can not be sure that the concentration of \(C. pneumoniae\) in the cell cultures corresponds to the concentration in the aortic wall in AAA disease. No quantitative studies have to our knowledge been performed. As the aortic samples were taken from autopsies, postmortem autolysis may account for some elastin degradation. However, the use of two adjacent aortic biopsies in each paired sample, minimizes the influence of postmortem autolysis.

Some direct and indirect mechanisms in vitro may be hypothesised and discussed. It has been reported that the surface of \(Chlamydia psittaci\) elementary body harbours a unique protease.\(^{29}\) It is possible that \(C. pneumoniae\) harbours a similar protease that can account for the increased elastin degradation in our experiment. \(C. pneumoniae\) may possibly induce the HEP-2 cells to express proteinases or cause activation of already expressed proteinases, which in turn may explain the enhanced elastin degradation. Additionally, increased apoptosis in \(C. pneumoniae\) infected HEp-2 cell cultures may cause an increase in proteinases in the medium.

**Discussion**

It is an intriguing hypothesis that \(C. pneumoniae\) by its presence in the aortic wall may contribute to the initiation and progression of AAA disease. It has been reported that serologic evidence of past infection with \(C. pneumoniae\) is associated with AAA disease,\(^{27}\) and an increased expansion rate of AAA is correlated to serologic evidence of a possible chronic \(C. pneumoniae\) infection.\(^{28}\) These observations may support the hypothesis.

Since elastin degradation is a key event in the development of an AAA and \(C. pneumoniae\) is commonly present in the aortic wall, it was tempting to investigate a possible association between elastin degradation and \(C. pneumoniae\). Our result show an increased degradation of elastin in aortic wall samples incubated in a \(C. pneumoniae\) infected cell culture compared with uninfected cell cultures. However, there were increases in EDP in the conditioned media in only 10 out of the 18 paired samples studied. This observation can not be explained in this study, but speculations on variations between cell cultures, such as the degree of apoptosis or the concentration of \(C. pneumoniae\) may be raised. Additionally, variations, possibly genetic in origin, in enzymes or connective tissue types between the aortic samples, may influence the result. Defects in the cross-linking of mature elastin may increase the susceptibility to degradation.

Although the in vitro mechanisms discussed earlier may not be directly applied to in vivo conditions, some effects of a chlamydial infection may explain enhanced elastin degradation even in vivo. Genital \(Chlamydia trachomatis\) infection significantly enhances granulocyte-elastase in semen,\(^{30}\) suggesting that \(C. trachomatis\) is a potent inflammatory inducer or induces increased elastase expression in granulocytes. \(C. pneumoniae\) may have a similar effect. A number of cytokines are increased in the wall of AAA\(^{31,32}\) and cytokines may be critical in the host defense reaction against intracellular bacteria. A persistent infection may contribute inflammation and fibrosis to the disease process.\(^{33}\) The release of elastin degradation products (EDP) in the extracellular matrix of the aortic wall may contribute to the inflammatory reaction, as EDP is chemotactic for neutrophils and monocytes.\(^{34}\) Additionally, EDP can stimulate smooth muscle cells to secrete elastase\(^{35}\) and an autonomous degenerative process may have started. Genetic variations in the response to an intracellular infection, or in the susceptibility to degradation of the structural proteins in
the aortic wall, may be critical for the type of late manifestation of aortic atherosclerosis.

*Chlamydia pneumoniae* infections in humans are very common,26,27 causing airway infections in both children and adults.26,28 Although these infections are common, *C. pneumoniae* is rarely detected in normal arteries,17 and is therefore not a frequent finding in other vascular lesions than atherosclerosis.29,30

AAA may be regarded as a late manifestation of a pathological process in the aortic wall. Traditionally, AAA has been considered to be atherosclerotic in origin, but recently attention has been drawn to the fact that patients with AAA are dissimilar to patients with aortic atherosclerotic occlusive disease and that atherosclerosis in AAA may be coincidental.40-43 However, it has been demonstrated that arteries increase in external size in response to the development of atherosclerotic plaques,44 and animal experiments show that atherosclerotic plaques,44 and animal experiments show that atherosclerosis may be a possible role played by *C. pneumoniae* in the early stages of AAA development.

In conclusion, the present investigation suggests that there is an association between the presence of *C. pneumoniae* and elastin degradation in the aortic wall in vitro. Consequently, *C. pneumoniae* may directly or indirectly play an active role in the degradation of elastin in the aortic wall. This finding encourages further investigations of *C. pneumoniae* in AAA disease.

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