 10.1111/j.1469-0691.2004.00920.x

Effect of chronic *Pseudomonas aeruginosa* infection on the development of atherosclerosis in a rat model

C. Turkay[1], R. Saba[2], N. Pahin[3], H. Altunbap[4], Ö. Özbudak[5], B. Akkaya[6], G. Özbilim[6], Ý. Gölbapý[1], M. Turkay[7], D. Öðünç[8] and Ö. Bayezid[1]

Departments of [1]Cardiovascular Surgery, [2]Infectious Diseases, [3]Anaesthesiology, [4]Endocrinology, [5]Chest Diseases, [6]Pathology, [7]Public Health and [8]Microbiology, Akdeniz University, Antalya, Turkey

ABSTRACT

In order to investigate the possible relationship between atherosclerosis and chronic *Pseudomonas aeruginosa* infection, 66 Wistar rats were given five separate intratracheal inoculations of either *P. aeruginosa* or sterile saline at 4-week intervals. The rats were divided into four groups: group 1 was infected with *P. aeruginosa* and fed a diet containing cholesterol 1% w/v; group 2 was infected with *P. aeruginosa* and fed a normal diet; group 3 was not infected and was fed a diet containing cholesterol 1% w/v; and group 4 (the control group) was not infected and was fed a normal diet. One month after the final inoculation, the rats were killed humanely; computerised image analysis was used to evaluate sections of the aorta and heart, and the maximal wall thickness of the aorta and coronary artery. The aortic wall thickness was significantly greater for group 1 (329.53 ± 58.06 μm) compared to groups 2 (190.59 ± 27.81 μm; $p < 0.0001$), 3 (262.90 ± 61.12 μm; $p < 0.0004$) and 4 (158.00 ± 30.30 μm; $p < 0.0001$). Similarly, the coronary artery wall thickness was significantly greater for group 1 (72.96 ± 10.67 μm) compared to groups 2 (35.07 ± 8.53 μm; $p < 0.0001$), 3 (41.45 ± 10.22 μm; $p < 0.0001$) and 4 (32.30 ± 5.27 μm; $p < 0.0001$). These findings strengthen the hypothesis that chronic infection plays a role in the pathogenesis of atherosclerosis.

Keywords Atherosclerosis, chronic infection, pathogenesis, *Pseudomonas aeruginosa*, rat model

Original Submission: 6 June 2003; **Revised Submission:** 25 September 2003; **Accepted:** 8 December 2003

Clin Microbiol Infect 2004; 10: 705–708

INTRODUCTION

Atherosclerotic cardiovascular disease is a major health problem throughout the world [1]. Although certain risk factors associated with the development of atherosclerosis have been defined, the exact mechanisms by which they might contribute to the development of atherosclerosis are not known fully [2] and many of the risk factors remain unexplained. Exposure to infectious agents has been proposed as a possible risk factor [3], and previous studies have demonstrated the possible role of several bacteria and viruses in the development of atherosclerosis [4–6]. Although infection can affect the atherosclerotic process directly by inducing a local inflammatory reaction associated with oxidative and proteolytic processes and proliferative cell responses, the indirect effects from distant sites which induce cytokines and systemic inflammation remain unknown. In the case of atherosclerosis, risk factors such as smoking, hypertension, hyperglycaemia, hyperlipidaemia and hypercholesterolaemia interact synergically. The objective of the present study was to determine whether chronic lung infection with *Pseudomonas aeruginosa* would result in the development of atherosclerosis in rats fed either a normal or cholesterol-supplemented diet.

MATERIALS AND METHODS

Bacteria

P. aeruginosa strain ATCC 1942, which has a stable mucoid phenotype, was used in the study. The bacteria were grown at 37°C for 18–24 h on sheep blood agar. The cells were then scraped from the agar and resuspended in sterile saline to a density of 5× McFarland standard (OD_{550} 1.25; equivalent to

Corresponding author and reprint requests: C. Turkay, Cardiovascular Surgery, Akdeniz University, Antalya, Turkey.
E-mail: turkey@med.akdeniz.edu.tr

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases

c. 1.5×10^9 CFU/mL). The viable count was confirmed by plating serial dilutions on sheep blood agar and counting colonies.

Experimental animals and study design

Sixty-six Wistar rats (aged 3 months; pathogen-free) were used. The rats were divided randomly into four groups, with 14–20 rats/group. The rats in group 1 (20 rats; four rats died during the experiment) and group 3 (16 rats; two rats died) were fed a diet supplemented with cholesterol (Sigma, St Louis, MO, USA) 1% w/v, while the rats in group 2 (14 rats; four rats died) and group 4 (16 rats; control group) were fed a normal diet. Each rat's trachea was surgically explored under anaesthesia with titrated intramuscular doses of ketamine hydrochloride (30–100 mg/kg) and xylazine hydrochloride (10–15 mg/kg). Rats in groups 1 and 2 were given 0.1 mL (1.5×10^9 CFU/mL) of *P. aeruginosa* suspension intratracheally, while the rats in groups 3 and 4 were given 0.1 mL sterile saline via a syringe. This procedure was repeated five times at 4-week intervals. While the rats were anaesthetised, a blood sample was collected from the tail (0.5 mL) for cholesterol analysis. Food consumption and weight were recorded each month. Animal care and processing was performed with strict adherence to the guidelines of the Institutional Animal Care and Use Committee.

Six months after the initial inoculation, all rats were killed humanely for evaluation of the aortic and coronary arteries. Following opening of the chest cavity under sterile conditions, the lungs were first removed and prepared for bacteriological examination, after which the aorta and heart were removed and sent for histopathological examination.

Bacteriological examination

Lung homogenates were prepared by adding an equal volume of phosphate-buffered saline to each of the lung tissue samples. Following homogenisation, 0.1-mL aliquots of appropriate dilutions were used to determine the viable count.

Histopathological examination

All histopathological examinations were performed blind by the pathological team. Representative cross-sections of the heart, and the thoracic and abdominal aortas, were removed from each sample and fixed in buffered formalin 10% v/v, embedded in paraffin wax, and stained with haematoxylin and eosin. The aorta was divided into three segments (ascending, descending and abdominal), and 5-μm-thick serial sections were prepared with a cryotome (Shandon AS 325; Thermo Electron, Runcorn, UK). Between eight and ten slides prepared from each segment were inspected visually to select the segment with greatest luminal narrowing, which was then examined with a CH 40 microscope (Olympus, Tokyo, Japan). Finally, the areas of media and the neointima were assessed by computerised image analysis (Samba 2000; Gateway Computers, Dublin, Ireland). Three slides with maximal aortic wall thickness were identified, and three pre-specified measurements, including intima plus media, were made by computerised image analysis for each rat. The microscopic and computerised image analyses were used to measure the epicardial coronary artery wall thickness in the heart sections. Three pre-specified measurements, including intima plus media, were made, and averages of the three measurements for each rat's aorta and coronary artery were used in the statistical analysis.

Statistics

Serum total cholesterol levels, food consumption and weights of the rats were expressed as mean ± SD. Food consumption and the weights of the groups were estimated by analysis of variance in repeated measures. Maximal aorta and coronary artery wall thickness, as well as serum total cholesterol levels, were also evaluated by analysis of variance, with a p value ≤ 0.05 considered significant.

RESULTS

Fifty-six rats survived for examination at the end of the study. There were no statistically significant differences in food consumption and weight between the groups. The total serum cholesterol levels of the rats in groups 1, 2, 3 and 4 were 78.76 ± 7.60 mmol/L, 61.10 ± 8.71 mmol/L, 86.68 ± 16.30 mmol/L and 74.75 ± 12.20 mmol/L, respectively. The rats in group 2 (infected group with a normal diet) had significantly less serum cholesterol ($p < 0.0001$) than groups 1, 3 and 4. *P. aeruginosa* was isolated from the lungs of seven rats in group 1, and three rats in group 2, but not from the other groups.

The rats in the control group had mainly normal aortic and coronary artery wall structure in cross-section. However, the rats in the infected group with a high-cholesterol diet had developed typical atherosclerosis lesions, not only in the aorta but also in the epicardial coronary arteries. In uninfected rats fed a high-cholesterol diet, there were marked atherosclerotic lesions in the aorta alone. Cross-sectional analysis revealed lesions that were uniformly characteristic of atheromas, with fatty streaks, various proportions of foamy cells, smooth muscle cells and extracellular matrix.

The aortic wall thickness was significantly greater for the infected group fed a high-cholesterol diet (329.53 ± 58.06 μm) as compared with the uninfected rats fed a high-cholesterol diet (262.90 ± 61.12 μm; p 0.004), the infected rats fed a normal diet (190.59 ± 27.81 μm; $p < 0.0001$), and the rats in the control group (158.00 ± 30.30 μm; $p < 0.0001$).

The coronary artery wall thickness was significantly greater in the infected group fed a high-cholesterol diet (72.96 ± 10.67 μm) as compared with the uninfected rats fed a high-cholesterol

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 705–708

diet (41.45 ± 10.22 μm; $p < 0.0001$), the infected rats fed a normal diet (35.07 ± 8.53 μm; $p < 0.0001$), and the rats in the control group (32.30 ± 5.27 μm; $p < 0.0001$). There was no significant difference between uninfected rats fed a high-cholesterol diet, infected rats fed a normal diet, and the control group.

DISCUSSION

Chronic infection has been associated previously with the development of atherosclerosis [2], raising the possibility that infectious agents may directly or indirectly trigger the cascade of biological and biochemical reactions leading to inflammation, atherosclerosis and vascular thrombotic events. Infection may cause lethal lytic damage, or infected cells may survive but show altered function [7]. Endothelial dysfunction may cause increased procoagulant activity, cytokine production and leucocyte adhesion, and reduced intrinsic fibrinolysis. Smooth muscle cell dysfunction may be associated with increased proliferation, reduced apoptosis, increased cholesterol esterification and increased cytokine production. Indirect effects on vascular cells may accompany infection or activation of vessel-associated leukocytes; mononuclear phagocytes may show increased procoagulant and reduced fibrinolytic behaviour, altered lipid metabolism, increased cytokine production and the release of toxic oxygen species [8]. Furthermore, it can be speculated that infectious agents may have direct and indirect effects on vascular cells.

After the discovery of the link between infection and atherosclerosis, several groups have developed animal models for further study. Muhlestein *et al.* [9] showed in a rabbit model that infection with *Chlamydia pneumoniae* may accelerate the development of atherosclerosis, and that treatment with azithromycin can prevent this process. With an *apoE*-deficient mouse model it has been shown that murine γ-herpes viruses and cytomegalovirus can accelerate atherosclerosis [10,11]. In mice fed a high-cholesterol diet, *C. pneumoniae* strain AR39 may stimulate the initial atherosclerotic lesions on vessels, although this is not the case with *Chlamydia trachomatis* strain MoPn [12].

Although the study of Hu *et al.* [12] suggested that *C. pneumoniae* may possess a unique biological property for its role in stimulating atherogenesis, the present study investigated a bacterium that causes chronic infection in other systems, namely a strain of *P. aeruginosa* with a mucoid phenotype. Such organisms have not been isolated previously from the aortic wall or the coronary artery wall. However, *P. aeruginosa* is one of the pathogens that is isolated most frequently from patients with chronic pulmonary infections, including cystic fibrosis. When grown on agar, *P. aeruginosa* isolates from chronically infected patients generally have a mucoid appearance. Examination of post-mortem lung material from patients with cystic fibrosis shows that formation of the glycocalyx of *Pseudomonas* is an in-vivo as well as an in-vitro phenomenon [13]. The exopolysaccharide produced by mucoid strains of *P. aeruginosa* mediates attachment to epithelial cells and mucins. In addition, the exopolysaccharide appears to protect the bacterium from host immune factors such as phagocytic cells [13]. Cash *et al.* [14] developed a model of chronic bronchopulmonary infection in rats with agarose beads embedded with *P. aeruginosa*. However, for the present study, it was decided to administer the organism repeatedly, as was done with the rat model of *C. pneumoniae* infection [9]. Technically, repeated intratracheal inoculation of bacteria enmeshed in agar beads to the same location could lead to airway obstruction, while direct tracheal inoculation of free *P. aeruginosa* has been shown to result in an acute or a transient pulmonary infection [15]. Although rats challenged with free live *P. aeruginosa* experienced mild-to-moderate lung pathology in comparison with rats challenged with *P. aeruginosa* alginate beads, their antibody responses were comparable, and the immunological responses to the antigens used were persistent during a 28-day study period [15]. Yu *et al.* [16] have used repeated aerosol administration successfully in attempts to reproduce the acquisition and pathogenesis of *P. aeruginosa* infection seen in cystic fibrosis patients [16].

Using the repeated administration method, the infected group of rats fed a normal diet had a significantly lower serum cholesterol level compared to the other groups. Studies in experimental animals, as well as in humans, have reported a decrease in cholesterol levels during infectious disease [17–19]. Although the underlying mechanism for this is unclear, several studies have suggested that cytokines can modulate lipid metabolism [17,18]. Elevated levels of cytokines such as

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 705–708

tumour necrosis factor are associated with hypocholesterolaemia [17]. It is unclear whether this decrease is caused by accelerated clearance of lipoproteins or diminished synthesis and secretion of lipoprotein precursors by the liver.

The present study is the first to demonstrate that infection with *P. aeruginosa* can stimulate atherogenesis in rats fed a cholesterol-supplemented diet. Infection with *P. aeruginosa* alone did not induce atherosclerotic lesions. In addition, the thickness of the coronary artery wall and the aorta wall was significantly greater in infected rats fed a cholesterol-supplemented diet, compared with the other rats. These findings strengthen the case for the role of chronic infection in the pathogenesis of atherosclerosis.

ACKNOWLEDGEMENTS

We would like to thank Medical Veterinarian Ş Atalay (Akdeniz University Experimental Animal Laboratory) for his assistance in the rat intra-tracheal inoculation during the initial stage of the study, and K. Bingam for her linguistic revision of the manuscript.

REFERENCES

1. US Department of Health and Human Services. *National Heart, Lung and Blood Institute fact book: Fiscal Year 1995*. Bethesda, MD: US Department of Health and Human Services, 1996.
2. Muhlestein JB. Coronary infection and coronary artery disease. *Med Clin North Am* 2000; **84**: 123–147.
3. Zhu J, Quyyumi AA, Norman JE *et al*. Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. *Am J Cardiol* 2000; **85**: 140–146.
4. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? *Lancet* 1997; **350**: 430–436.
5. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation* 1999; **100**: 20–28.
6. Leinonen M, Saikku P. Evidence for infectious agents in cardiovascular disease and atherosclerosis. *Lancet Infect Dis* 2002; **2**: 11–17.
7. Libby P, Egan D, Skarlatos S. Roles of infectious agents in atherosclerosis and restenosis. *Circulation* 1997; **96**: 4095–4103.
8. Anderson JL, Muhlestein JB, Carlquist JF *et al*. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for *Chlamydia pneumoniae* infection. *Circulation* 1999; **99**: 1540–1547.
9. Muhlestein JB, Anderson JL, Hammond EH *et al*. Infection with *Chlamydia pneumoniae* accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation* 1998; **97**: 633–636.
10. Hisich E, Zhou YF, Paigen B, Johnson TM, Burnett MS, Epstein SE. Cytomegalovirus infection increases development of atherosclerosis in apolipoprotein-E knockout mice. *Atherosclerosis* 2001; **156**: 23–28.
11. Alber DG, Powell KL, Vallance P, Goodwin DA, Grahame-Clarke C. Herpesvirus infection accelerates atherosclerosis in the apolipoprotein E-deficient mouse. *Circulation* 2000; **102**: 779–785.
12. Hu H, Pierce GN, Zhong G. The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. *J Clin Invest* 1999; **103**: 747–753.
13. Pollack M. *Pseudomonas aeruginosa*. In: Mandell GL, Bennett JE, Dolin R, eds. *Principles and practice of infectious diseases*, 5th edn. New York, NY: Churchill Livingstone, 2000; 2310–2335.
14. Cash HA, Woods DE, McCullough B, Johanson WG, Bass JA. A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am Rev Respir Dis* 1979; **119**: 453–459.
15. Johansen HK, Espersen F, Pedersen SS, Hougen HP, Rygaard K, Hoiby N. Chronic *Pseudomonas aeruginosa* lung infection in normal and athymic rats. *APMIS* 1993; **101**: 207–225.
16. Yu H, Hanes M, Chrisp CP, Boucher JC, Deretic V. Microbial pathogenesis in cystic fibrosis. Pulmonary clearance of mucoid *Pseudomonas aeruginosa* and inflammation in a mouse model of repeated respiratory challenge. *Infect Immun* 1998; **66**: 280–288.
17. Fraunberger P, Guenther P, Cremer P, Werdan K, Walli AK. Association of serum tumor necrosis factor levels with decrease of cholesterol during septic schock. *Shock* 1998; **10**: 359–363.
18. Sammalkorpi KT, Valtonen VV, Kertulla Y, Nikkila E, Taskinen NR. Changes in serum lipoprotein pattern induced by acute infections. *Metabolism* 1988; **37**: 859–865.
19. Fiser RH, Enniston JC, Beisel WR. Infection with *Diplococcus pneumonia* and *Salmonella typhimurium* in monkeys: changes in plasma lipids and lipoproteins. *J Infect Dis* 1972; **125**: 54–60.

© 2004 Copyright by the European Society of Clinical Microbiology and Infectious Diseases, *CMI*, **10**, 705–708