The association between the development of renal artery atherosclerosis and chronic *Pseudomonas aeruginosa* infection in rats

Sıçanlarda renal arterde ateroskleroz gelişimiyle kronik Psödomonas aeruginosa infeksiyonu arasındaki ilişki

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Background: In this study we investigated the role of chronic lung infections induced by *Pseudomonas aeruginosa (P. aeruginosa)* in a rat model in the development of renal artery atherosclerosis.

Methods: Sixty-six Wistar albino rats were randomly divided into four groups each consisting of 14 to 20 rats. The rats in group 1 (n=20) and group 3 (n=16) were fed with a 1% cholesterol supplemented diet, whereas the rats in group 2 (n=14) and group 4 (Control group) were fed with regular rat chow. Rats' tracheas were surgically explored under anesthesia. Intratracheal inoculation was performed with *P. aeruginosa* suspension in group 1 and 2 or 0.1 ml saline in groups 3 and 4 via a syringe five times with four-week-intervals during the study period. Cholesterol levels were measured in blood samples collected from the tail under anesthesia.

Results: In histopathological examinations; the segment with greatest luminal narrowing was selected by visual inspection and 8-10 slides prepared from each segment were examined under Olympus CH 40 microscope. Medial and the neointimal areas of the specimens were analyzed by computed image analysis.

Conclusion: The rats in the control group exhibited mainly normal renal artery wall structure on cross sections. The rats that were infected and fed with 1% cholesterol diet developed typical preatherosclerotic changes in the renal arteries. In this study, it was demonstrated for the first time that both chronic lung infection with *P. aeruginosa* and high cholesterol feeding accelerated the increases of the renal artery intima-media thickness in a rat model. These findings strongly suggest that the distant effects of chronic infection are an etiological factor in genesis of atherosclerosis.

Key words: Atherosclerosis; *Pseudomonas aeruginosa*; rat; renal artery.

Amaç: Bu çalışmada *Psödomonas aeruginosa (P. aeruginosa)* ile sıçan modelinde oluşturulan kronik akciğer infeksiyonlarının renal arterde ateroskleroz oluşumundaki rolü araştırıldı.

Çalışma planı: Altmış altı Wistar albino sıçan her grupta 14 ile 20 adet olacak şekilde randomize olarak dört gruba ayrıldı. Grup 1 (n=20) ve grup 3 (n=16) %1 kolesterol eklenmiş diyet ile, grup 2 (n=14) ve grup 4 (Kontrol grubu) standart sıçan yemi ile beslendi. Sıçanların trakeaları anestezi altında cerrahi olarak çıkarıldı. Çalışma periyodu esnasında dört haftalık aralıklarla toplam beş defa bir enjektörle grup 1 ve 2'ye *P. aeruginosa* süspansiyonu ile grup 3 ve 4'e ise serum fizyolojik ile intratrakeal inokülasyon yapıldı. Kolesterol seviyeleri anestezi altında kuyruktan alınan kan örneklerinden değerlendirildi.

Bulgular: Histopatolojik incelemelerde en fazla luminal incelme olan segment gözle inceleme yoluyla seçildi ve her segmentten 8-10 kesit hazırlanarak Olympus CH 40 mikroskop ile incelendi. Örneklerin medial ve neointimal bölgeleri bilgisayarlı görüntü analizi ile analiz edildi.

Sonuç: Kesitler incelendiğinde kontrol grubundaki sıçanların esas olarak normal renal arter duvarı yapısına sahip olduğu görüldü. İnfekte edilen ve %1 kolesterol eklenmiş diyet ile beslenen grupta renal arterlerde tipik preaterosklerotik değişiklikler gelişti. Bu çalışmada bir sıçan modelinde *P. aeruginosa* ile oluşturulan kronik akciğer infeksiyonu ve yüksek kolesterol ile beslenmenin renal arter intima-media kalınlığını artırdığı ilk defa olarak gösterildi. Bu bulgular kronik infeksiyonun uzak etkilerinin ateroskleroz oluşumunda bir etyolojik faktör olduğunu güçlü şekilde düşündürmektedir.

Anahtar sözcükler: Ateroskleroz; *Psödomonas aeruginosa*; sıçan; renal arter.

Received: December 1, 2009 *Accepted*: March 23, 2010

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Atherosclerosis is a disease of elastic arteries (i.e. aorta, carotid and iliac arteries) and large and medium-sized muscular arteries (i.e. coronary and popliteal arteries) whereas smaller arteries rarely became affected. It is a focal disease that does not affect arteries uniformly. The focal nature of the disease is in apparent contrast to the fact that most risk factors for development of atherosclerosis, such as hyperlipidemia, hypertension, smoking and diabetes mellitus, are systemic and are likely to affect all parts of the arterial system similarly. This clearly shows that the systemic risk factors must act in concert with local factors. One such factor is the local shear stress exerted by blood flow. However, apparent risk factors cannot be determined in half of the cases with atherosclerosis. Therefore there is debate in the pathogenesis of atherosclerosis.[1-2]

More recently attention has focused on the role of microorganisms in atherosclerosis. Several epidemiologic studies have demonstrated an association between *Chlamydia pneumonia (C. pneumonia)* infection and cardiovascular disease. Infection can affect the atherosclerotic process directly by inducing a local inflammatory reaction associated with oxidative and proteolytic process and proliferative cell responses, the indirect effects from distant sites by inducing cytokines and systemic inflammation is a question of debate.[3,4] There is little knowledge of the role of infection in the pathogenesis of renal artery atherosclerosis.[5]

This study explores a possible relationship between renal artery atherosclerosis and chronic lung infection with *Pseudomonas aeruginosa (P. aeruginosa)* in a rat model. The objectives of the present study were to determine whether chronic lung infection with *P. aeruginosa* in rats fed with regular rat diet (Chow) or 1% cholesterol-rich diet would result in atherosclerosis.

MATERIALS AND METHODS

Bacteria

In this study we used *P. aeruginosa* American type culture collection (ATCC) 1942 which stably maintains a mucoid phenotype. The concentration of the bacteria was prepared turbidometrically after it was inoculated at 37 °C for 18-24 hours in sheep blood agar and finally adjusting the concentration to 1.5x109 colony forming unit (CFU)/ml. The concentrations were confirmed by plating serial dilutions on the appropriate culture medium and counting colonies were used to confirm the concentrations.

Experimental animals and study design

Three-month-old, pathogen-free Wistar albino rats (n=66) were used. The rats were randomly divided into four groups having fourteen to twenty rats in each group. The rats in group 1 (n=20;) and group 3 (n=16) were fed with a 1% cholesterol-supplemented diet (Sigma, St. Louis, MO63178, USA), whereas the rats in group 2 (n=14) and group 4, the control group, were fed with regular rat chow. Four rats in group 1, two rats in group 3 and four rats in group 2 died during the experiment. Using titrated intramuscular doses of ketamine hydrochloride (30-100 mg/kg) and xylazine hydrochloride (10-15 mg/kg) the rats' tracheas were surgically explored under anesthesia and 0.1 ml (1.5x109 CFU/ml) of *P. aeruginosa* suspension (in group 1 and 2) or 0.1 ml saline (in groups 3 and 4) were given intratracheally via a syringe five times at four-week-intervals during the study period. Cholesterol levels were measured by a blood sample collected from the tail (0.5 ml) under anesthesia. Animal chow consumption and weights were recorded monthly. Animal care and processing were performed under strict adherence to the Institutional Animal Care and Use Committee guidelines.

After six months from the first inoculation the rats were sacrificed to proceed to evaluate the renal arteries. First, the chest cavities were opened and the lungs removed under sterile conditions, and half of the lungs were prepared for bacteriological examination. Secondly, after opening abdominal cavities, the renal arteries were excised from the point that renal arteries join the aorta. The renal arteries were sent for histopathological examination.

Bacteriological examination

Samples were appropriately diluted and plated to determine the numbers of CFU. To prepare the bacterial sample 0.1 ml of the homogenate was plated. Equal volumes of phosphate-buffered saline were added to each of the lung tissue samples, and the mixture was then homogenized to prepare the lung homogenates.

Histopathological examination

The pathology team that performed histopathological examinations was blinded to the specimens. The team first grossly inspected the specimens. Representative cross-sections of the renal arteries were prepared as follows: histological specimens were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Five-micrometer(μm)-thick serial sections were prepared using cryotome (Shandon AS 325, Cheshire, WA7 1PR, England) after cross-sections of the specimen from the aorta-renal artery junction and middle of the renal artery were removed from each sample. The segment with greatest luminal narrowing was selected by visual inspection of the 8-10 slides prepared from each segment and then examined by Olympus CH 40 microscope (Olympus

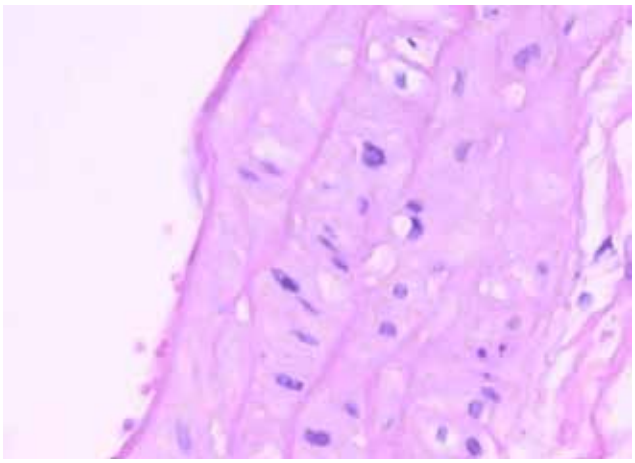

Fig. 1. Renal artery wall structure on cross sections in the control group (H-E x 200).

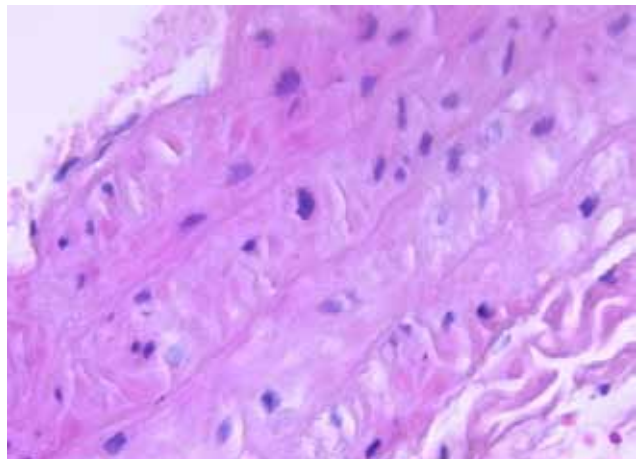

Fig. 2. Renal artery wall structure on cross sections in the infected plus fed with 1% cholesterol diet group (H-E x 200).

optical Co, Ltd, Japan). Media and the neointima regions of the specimens were analyzed by computed image analysis (Samba 2000, Gateway, GP7-450, GW-2K, Ireland). After determining three slides having maximal renal artery wall thickness, computed image analysis was used to perform three pre-specified measures including intima plus media and an average of the three measurements for each section were used in the statistical analysis.

Statistics

Serum total cholesterol levels, chow consumption and the weights of the rats were expressed as mean±standard deviation. Chow consumptions and the weights of the groups were estimated by analysis of variance in repeated measures. In addition maximal renal artery wall thickness, serum total cholesterol levels were evaluated by variance analysis and the p-value ≤0.05 was considered significant.

RESULTS

Fifty-six rats survived and could be examined. During this study, there were statistically no differences in chow consumption and weight among 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, 74.75±12.20 mmol/l, respectively. The rats in group 2 (infected-only group) had significantly less serum cholesterol level than the groups 1, 3 and 4 ($p<0.0001$). *Pseudomonas aeruginosa* was isolated from the lungs of the seven rats in group 1 and the three rats in group 2 but none from the other groups.

The rats in the control group exhibited mainly normal renal artery wall structure on cross sections (Fig. 1). However, the rats in the infected plus 1% cholesterol diet-fed group developed typical preatherosclerotic lesions in the renal arteries. Cross sectional analysis revealed that lesions were uniformly characteristic of

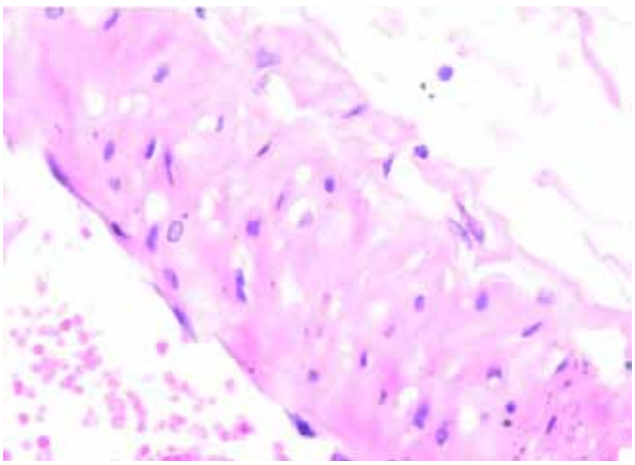

Fig. 3. Renal artery wall structure on cross sections the rats fed 1% cholesterol-diet alone (H-E x 200).

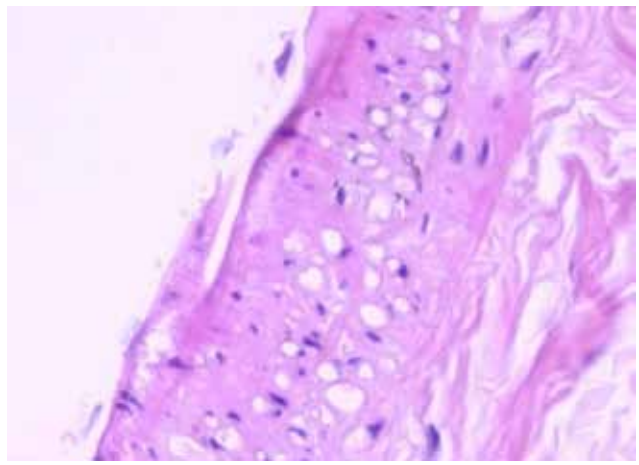

Fig. 4. Renal artery wall structure on cross sections the only infected rats (H-E x 200).

preatherosclerotic lesions with fatty streaks, various proportions of foamy cells, smooth muscle cells and extracellular matrix (Fig. 2).

The intima-media thickness at the middle of the right renal artery had significantly increased in the infected plus 1% cholesterol diet-fed rats (70.04±4.46 μm) when compared with the rats fed 1% cholesterol diet alone (63.94±3.34 μm; p<0.001; Fig 3), the infected-only rats (59.83±1.72 μm; p<0.001; Fig 4), and the rats in the control group (56.51±1.53 μm; p<0.001).

The intima-media thickness at the middle of the left renal artery had significantly increased in the infected plus fed 1% cholesterol-diet rats (71.19±4.13 μm) when compared with the rats fed 1% cholesterol-diet alone (64.20±4.35 μm; p<0.001), the only infected rats (60.20±1.21 μm; p<0.001), and the rats in the control group (55.26±1.48 μm; p<0.001).

The intima-media thickness at the junction of the right renal artery aorta had significantly increased in the infected plus fed 1% cholesterol-diet rats (96.97±4.13 μm) when compared with the rats fed 1% cholesterol-diet alone (88.52±2.34 μm; p<0.001), the only infected rats (88.29±2.35 μm; p<0.001), and the rats in the control group (74.21±2.64 μm; p<0.001).

The intima-media thickness at the junction of the left renal artery aorta had significantly increased in the infected plus 1% cholesterol diet-fed rats (97.54±5.25 μm) when compared with the rats fed 1% cholesterol diet alone (89.18±2.49 μm; p<0.001), the infected-only rats (89.49±1.25 μm; p<0.001), and the rats in the control group (74.13±2.54 μm; p<0.001).

DISCUSSION

Renovascular disease accounts for 1-2% of adult cases of secondary hypertension. Most renovascular hypertension develops from partial obstruction of the main renal artery. The partial obstruction of a main renal artery in adult patients with renovascular hypertension is most commonly caused by atherosclerosis. Atherosclerotic disease is more preponderant in older men, in whom it accounts for about two-thirds of the cases.[6,7] This study showed that both chronic lung infection with *P. aeruginosa* and high cholesterol/high fat feeding accelerated the increases of the renal artery intima-media thickness in a rat model. Also, acceleration in the increases of intima-media thickness with chronic lung infection with *P. aeruginosa* showed in high cholesterol/high fat feeding rats.

It has been shown that atherosclerotic lesions in the hyperlipidemic swine model almost exclusively arise from intimal thickening point, and accelerated increases in the intima-media thickness are important in the genesis of atherosclerosis.[8]

Intima-media thickness of the carotid arteries can be measured noninvasively by using ultrasound techniques. Normally in healthy individuals there is a slow increase of the intima-media thickness with age.[9] Some studies showed that the increases in intima and media thickness are about three times faster in patients with vascular disease.[10] Intimal thickening and intimal xanthomas, which are accepted as preatherosclerotic lesions, are commonly produced in high cholesterol/high fat feeding animal models.[11] In this study we showed that increases in intima-media thickness in high cholesterol/high fat feeding rats were accelerated with chronic lung infection with *P. aeruginosa*.

There is little knowledge of the role of infection in the pathogenesis of renal artery atherosclerosis. Infection can affect the atherosclerotic process directly by inducing a local inflammatory reaction associated with oxidative and proteolytic process and proliferative cell responses, the indirect effects from distant sites by inducing cytokines and systemic inflammation. The role of infection in the genesis of atherosclerosis was investigated in several animal models. Muhlestein et al.[12] showed that infection with *C. pneumonia* may accelerate the development of atherosclerosis and treatment with azithromycin may prevent it in a rabbit model. With apoE-deficient mouse model it has been shown that both murine γ-herpes viruses and CMV accelerate atherosclerosis.[13,14] In mice fed the high cholesterol diet, *C. pneumonia* strain AR39 may stimulate the initial atherosclerotic lesions on vessels, however this is not the case with the *C. trachomatis* strain mouse pneumonitis (MoPn) organisms.[15] *C. pneumonia* was isolated from the atherosclerotic plaques. Therefore, *C. pneumonia* was suggested to possess a unique biologic property for its atherogenesis. But distant effects of infection were not clearly defined. In this study, we chose a non-specific bacterium *P. aeruginosa* (mucoid phenotype) leading to chronic infection in other systems to evaluate the distant effect of infection, as this microorganism has not been previously isolated from the aortic or other arterial walls.

P. aeruginosa is one of the most frequently isolated bacterial pathogens in patients with chronic pulmonary infections, including cystic fibrosis. The mucoid exopolysaccharide produced by mucoid strains form a matrix arround the bacterium, protecting it from host immune factors such as phagocytic cells.[16] Cash et al.[17] originally developed a model of chronic bronchopulmonary infection in rats using agarose beads embedded with *P. aeruginosa*. While planning the study we decided to employ repeated administration of the organism as it was done with the rat model of

C. pneumonia infection.[12] Direct tracheal inoculation of free *P. aeruginosa* have resulted in an acute or a transient pulmonary infection.[18] Although the rats challenged with free live *P. aeruginosa* experienced mild to moderate lung pathology as compared to the rats challenged with *P. aeruginosa* alginate beads, their antibody responses were comparable and the immunological responses to the antigens used were persistent during the whole 28 day study period.[18]

Finally, this study first demonstrated that both chronic lung infection with *P. aeruginosa* and high cholesterol/high fat feeding accelerated the increases of the renal artery intima-media thickness in a rat model. Renal artery intima-media thickness was significantly increased in the infected plus 1% cholesterol diet rats compared with the rats fed 1% cholesterol diet alone. These findings strengthened the opinion that the distant effects of chronic infection are an etiological factor in the genesis of atherosclerosis.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

The Akdeniz University Scientific Research Projects Unit, Antalya, Turkey supported this study.

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