Cadmium Accumulation in Aortas of Smokers
Shadi Abu-Hayyeh, Minder Sian, Keith G. Jones, Ari Manuel, Janet T. Powell

Abstract—Abdominal aortic aneurysm is a smoking-related disorder. Cadmium, inhaled from cigarettes, may accumulate in the aorta and facilitate weakening of the aorta through adverse effects on smooth muscle cell metabolism. Cadmium was measured by atomic absorption spectrometry in infrarenal aortas from 13 patients with abdominal aortic aneurysm and from 17 age- and sex-matched patients with normal-diameter abdominal aorta. Total cadmium content was associated with smoking, assessed as pack-years (r=0.54, P=0.004), but was similar in aneurysmal and undilated aortas. The cadmium content (mean±SE) was higher in the media (3.25±0.53 ng/mg dry wt, 7±1.2 μmol/L) than in the intima or adventitia (1.14±0.24 and 1.87±0.38 ng/mg dry wt, respectively; ANOVA, P<0.005). There was a strong correlation between medial cadmium content and pack-years of smoking (r=0.87, P<0.001). In aortic smooth muscle cells cultured on fibrillar collagen, cadmium inhibited DNA synthesis and collagen synthesis and diminished cell proliferation, compared with endothelial cells or fibroblasts, and this may result in a heightened sensitivity of cultured smooth muscle cells to cadmium toxicity. Previous reports have indicated that metallothionein is not well induced in smooth muscle cells cultured on fibrillar collagen type I. We also followed the synthesis of procollagen by these cells and the induction of metallothionein. Metallothionein is considered to have a key role in cellular mechanisms, in that it detoxifies cadmium, acts as an antioxidant under a variety of conditions, and has a role in NO signaling. Previous reports have indicated that metallothionein is not well induced in smooth muscle cells compared with endothelial cells or fibroblasts, and this may result in a heightened sensitivity of cultured smooth muscle cells to cadmium toxicity. In the presence of cadmium accumulation, similar mechanisms could be important in fully understanding how smoking damages the vasculature.

Key Words: aortas ■ aneurysm ■ smoking ■ muscle smooth ■ collagen

Cadmium is one of the many toxic components of inhaled tobacco smoke. The amount of cadmium inhaled from each pack of 20 cigarettes is ≈16 μg.1 Cadmium has a long elimination time (estimated at 10 to 30 years), providing the possibility for the accumulation of substantial amounts of cadmium during the lifetime of a smoker. There are reports that cadmium alters the metabolism of cultured human aortic smooth muscle cells and the processing of collagen.2,3 Cadmium also blocks calcium channels and inhibits ATPases and other ion transport systems.

Smoking is the most consistent risk factor for the development and expansion of abdominal aortic aneurysms (AAAs).4,5 The histological features of AAA include atherosclerosis, loss of medial smooth muscle (with evidence of apoptosis), and loss of elastin (with remodeling of the connective tissue). Therefore, we wished to investigate the hypothesis that cadmium, from cigarettes, accumulates in the infrarenal abdominal aorta, thus stimulating aneurysmal dilatation. To test this hypothesis, we compared the concentration of cadmium in infrarenal aortas from patients with and without AAAs.

After finding that the highest concentration of cadmium was in the medial layer of the aorta, we investigated whether similar concentrations of cadmium altered the metabolism of cultured aortic smooth muscle cells. Arterial smooth muscle cells have a low proliferative index in vivo and are surrounded by an extracellular matrix, which includes collagen (types I, III, and IV) and laminin.6 Interactions of the smooth muscle cells with the underlying matrix regulate cell proliferation, which may be dependent on proteolysis of the matrix and disruption of the integrin-dependent cell-matrix interactions.7 Therefore, to mimic the in vivo situation, we studied DNA synthesis and the proliferation of smooth muscle cells cultured on fibrillar collagen type I. We also followed the synthesis of procollagen by these cells and the induction of metallothionein. Metallothionein is considered to have a key role in cellular mechanisms, in that it detoxifies cadmium, acts as an antioxidant under a variety of conditions, and has a role in NO signaling.8,9 Previous reports have indicated that metallothionein is not well induced in smooth muscle cells compared with endothelial cells or fibroblasts, and this may result in a heightened sensitivity of cultured smooth muscle cells to cadmium toxicity.10 In the presence of cadmium accumulation, similar mechanisms could be important in fully understanding how smoking damages the vasculature.

Methods
Biopsies of abdominal aorta, from opposite the inferior mesenteric artery, were obtained with local ethical committee approval. Pipe and cigar smokers were excluded. The smoking history of patients was
obtained by ≥2 of the following methods: direct questioning, information from hospital records, and relative interviews. One pack-year was equivalent to smoking 20 cigarettes per day for 1 year. Patients included 13 undergoing AAA repair (AAA diameter 5.6 to 9.5 cm, mean age 71.6±9.3 years, 8 men and 5 women) and 17 with undilated aortas (1.6 to 2.2 cm) at postmortem examination (mean age 75.5±9.3 years, 10 men and 7 women). The biopsy was dissected along planes into intima, media, and adventitia, and the dissection was confirmed by histology. These aortic samples were dried to constant weight over P₂O₅ before hydrolysis, at room temperature for 24 hours, in concentrated HNO₃ (10 to 30 mg/mL).

Cadmium was measured by electrothermal atomization atomic absorption spectrometry with the use of a 5030 Atomic Absorption Spectrophotometer (Perkin-Elmer). Samples were diluted with a matrix modifier containing 600 mg/L palladium and 0.1% Triton X-100 and were adjusted to pH 9 with ammonia solution. Standard preparations of bovine liver or porcine kidney (Certified Reference Materials BCR185 and BCR186, from the Laboratory of the Government Chemist, London, UK), with known cadmium concentrations, were used for validation of the assay (coefficient of variation <10%).

Human fetal aortic smooth muscle cells were purchased from Clonetics and cultured in Ham's F-12K nutrient mixture (GIBCO-BRL), with Kaighn's modification containing 10 μg/mL insulin, 10 μg/mL transferrin, 10 mg/mL sodium selenite, 20 μg/mL endothelial cell growth supplement, and 10% FCS (all from Sigma). Fibrillar collagen-coated plastic was prepared as described previously. Polymerization was confirmed by observing the gelation of aliquots (50 μL) of the neutralized collagen solution (1 mg/mL) in a separate 96-well plate. The cultured smooth muscle cells were stained positively for smooth muscle cell markers, including smooth muscle cell–specific actin and myosin. Cells were seeded at 7500 cells per well at passages 14 to 18 on plastic ware coated with rat-tail type I collagen (Stratech). The cells were cultured in serum-rich medium for 24 hours and then growth-arrested in serum-free medium for 24 hours before the addition of cadmium in complete F-12K medium, with 4 replicate wells used for each concentration. After incubation of the cells with varying concentrations of cadmium for 24 hours, DNA synthesis (incorporation of bromodeoxyuridine with thymidine) was measured by colorimetric assay 13: each well at passages 14 to 18 on plastic ware coated with rat-tail type I collagen was prepared as described previously, with varying concentrations of cadmium for 24 hours, fixed with 4% formaldehyde at room temperature overnight, and washed 3 times with PBS before immunostaining. Cells, in a 96-well plate, were incubated with varying concentrations of cadmium for 24 hours, fixed with 4% formaldehyde at room temperature overnight, and washed 3 times with PBS before immunostaining. Cells were then incubated with a mouse monoclonal anti-metallothionein, recognizing MT-1 and MT-2, (Zymed Laboratories) diluted to 1:400 in PBS containing 10% FCS and 0.5% Tween 20, for 2 hours at 37°C. (The specificity of the primary antibody was confirmed by Western blotting: in lysates from confluent cells a single faint band at 6.5 kDa was observed.) After a wash, there were sequential incubations at room temperature with biotinylated goat anti-mouse IgG (1:1000, Zymed Laboratories) and streptavidin horseradish peroxidase (1:1000, Zymed Laboratories) before the development of peroxidase activity with o-phenylenediamine (Sigma), monitoring absorbance at 492 nm. Quadruplicate wells were used for each condition, and the cell number (by Picogreen assay) was estimated from an identical number (by Picogreen assay) was estimated from an identical

Figure 1. Cadmium content of aorta and smoking history. The cadmium content of aortic media increases with lifetime number of cigarettes smoked (Spearman correlation coefficient, r = 0.087, P < 0.001). Filled circles show cadmium content of aortic media from patients with AAA; open circles, data from patients with normal-diameter aorta. One AAA patient and 4 other patients had never smoked. All of the patients with a smoking history of >40 pack-years were current smokers. Smoking history is given as pack-years.

tallothionein antibody at 1:50 dilution, by the Department of Histopathology at Charing Cross Hospital.

Demographic data are reported as mean±SD, and cadmium data are reported as mean±SE. Statistical comparisons were made by use of Spearman correlation coefficients, Mann-Whitney U tests, and ANOVA.

Results

There was no difference in age, sex, or smoking habits between the patients with AAA and the control group with normal aortic diameters; mean pack-years of smoking were 30 and 27 years, respectively (Mann-Whitney test, P = 0.69).

The cadmium concentration of full-thickness aortas ranged from 0.01 to 6.73 ng/mg dry wt (mean 1.46±0.35 ng/mg dry wt). The medial layer always contained the highest concentration of cadmium (range 0.03 to 9.58 ng/mg dry wt). The concentrations of cadmium in the intimal, medial, and adventitial layers were 1.14±0.24, 3.25±0.53, and 1.88±0.35 ng/mg dry wt, respectively (ANOVA, P < 0.001). There was no difference in the cadmium content of aortic media samples between normal diameters (2.97±0.59 ng/mg dry wt) and aneurysmal diameters (3.65±1.0 ng/mg dry wt; Mann-Whitney, P = 0.53). Individual values are shown in Figure 1. Similarly, there were no differences between the cadmium content of full-thickness aortas in the 2 patient groups, and there was no correlation between either age or aortic diameter and cadmium content of any layer. However, there was a strong positive relationship between cadmium concentration and the pack-years of cigarettes smoked, for full-thickness aorta (r = 0.54, P = 0.004). A significant correlation between cadmium and pack-years of smoking was observed for all layers of the aorta but was strongest for the medial layer (r = 0.87, P < 0.001; Figure 1). The low cadmium content of aorta in those who had never smoked is evident.

For aortic media, the mean dry weight–to–wet weight ratio was 0.25±0.05. Therefore, the mean cadmium concentration in this layer was ≈7 μmol/L (range 0.01 to 20 μmol/L). Aortic smooth muscle cells were cultured with increasing concentrations of cadmium chloride (0 to 1000 μmol/L) for
24 hours. In the absence of cadmium, cells cultured on fibrillar collagen had procollagen synthesis rates (estimated from total hydroxyproline in the culture media) of 2.2±0.4 pmol hydroxyproline/µg DNA per hour (4 separate experiments). This reduced to 2.0±0.4, 1.7±0.6, 1.4±0.3, 1.3±0.5, and 1.2±0.2 pmol hydroxyproline/µg DNA per hour at cadmium concentrations of 1, 3, 10, 30, and 300 µmol/L, respectively (IC₅₀ 6.2 µmol/L, 4 separate experiments). There also was a concentration-dependent reduction in DNA synthesis (bromodeoxyuridine incorporation) and total cell number (Picogreen fluorescence), with IC₅₀ values of 2±1 µmol/L (5 separate experiments) and 6±2 µmol/L (4 separate experiments), respectively (Figure 2).

The basal staining for metallothionein in aortic smooth muscle cells, cultured on fibrillar collagen, was weak. After incubation of the cells with increasing concentrations of cadmium for 24 hours, a progressive increase in metallothionein (staining per 10 000 cells) was observed (Figure 3). The EC₅₀ for cadmium was 23±3 µmol/L.

Discussion

Most smokers develop extensive aortic atherosclerosis, and some of these develop AAA. We observed that aortic cadmium content increased with the number of pack-years smoked. However, because there was no association between cadmium content and aortic diameter, we failed to prove our hypothesis that selective accumulation of cadmium was associated with the development of AAA.

Cadmium, inhaled cigarette smoke, deposited in the aorta could take 10 to 30 years to be eliminated. Slow elimination of cadmium could alter the relationship between aortic cadmium content and pack-years of smoking. Smoking history is often not accurately reported and recorded. Nevertheless, we observed a strong correlation between aortic cadmium content and pack-years, with most cadmium being present in the medial layer of aorta. Loss of medial smooth muscle cells and excessive medial matrix proteolysis are hallmarks of aneurysm pathology. Therefore, it is possible that the response of medial aortic smooth muscle cells to cadmium could be subtly different for patients who develop AAA. The mechanisms whereby cadmium causes cell toxicity are not well understood. Cadmium inhibits L-type calcium channels (present in smooth muscle cells), reacts with thiol groups, may displace zinc from important metabolic enzymes, and is associated with the generation of oxygen-derived free radicals. These and other factors are likely to contribute to the relative concentration of cadmium in the medial layer of the aortic wall.

We calculated that the average cadmium concentration in the medial layer was 7 µmol/L. This is identical to the concentration of cadmium calculated to be in the lungs of patients with moderate to severe emphysema. This concentration of cadmium (7 µmol/L) was sufficient to significantly reduce the procollagen synthesis of aortic smooth muscle cells. The reduction in procollagen synthesis is concordant with previous findings for lung fibroblasts, in which cadmium concentrations of 10 to 30 µmol/L inhibited procollagen synthesis. Fibrellar collagen is present in the normal aortic media and is known to regulate integrin
signaling and the proliferation of vascular smooth muscle cells. When human aortic smooth muscle cells are cultured on fibrillar collagen, they proliferate, but they do so slowly because of the altered regulation of Cdk2 inhibitors. We also observed that aortic smooth muscle cells proliferate slowly when cultured on fibrillar collagen type I and that very low concentrations of cadmium (≤10 μmol/L) were sufficient to inhibit DNA synthesis and cell proliferation. Interestingly, these low concentrations of cadmium did not appear to be sufficient for the rapid upregulation of metallothionein in aortic smooth muscle cells cultured on fibrillar collagen. The increased expression of metallothionein has a critical role in protection against cadmium toxicity. Therefore, because cadmium accumulates in the aorta, the protective mechanism of increased metallothionein expression may occur only after extensive cellular damage has occurred. In the diseased aneurysmal aorta, metallothionein was expressed weakly in the remnant medial smooth muscle cells but was expressed strongly in inflammatory cells (Figure 4): the expression of metallothionein in the inflammatory cells is not necessarily related to cadmium toxicity. These sections represent end-stage disease, but the situation may be very different in early disease. However, the available evidence indicates that metallothionein is poorly expressed in human aortic smooth muscle cells. In contrast, another stress response gene, cyclooxygenase 2, also is expressed widely in smooth muscle and inflammatory cells in aneurysmal aortas.

The insensitive response of metallothionein expression to cadmium in human aortic smooth muscle cells was similar to that observed previously in bovine aortic smooth muscle cells. These effects also are likely to be dependent on cell-matrix interactions. We have preliminary evidence showing that cells cultured on plastic have much stronger induction of metallothionein at cadmium concentrations of ≤10 μmol/L and, conversely, are much less sensitive to growth inhibition by cadmium than are the slowly growing cells cultured on fibrillar collagen. In addition to the regulation by substratum, there may also be species differences. Others have reported the rapid upregulation of metallothionein within 24 hours of exposure to 5 μmol/L cadmium in porcine aortic smooth muscle cells. The change to a synthetic proliferative phenotype of aortic smooth muscle cells in culture is likely to limit the interpretations of findings from all cell culture experiments. However, culture of smooth muscle cells on fibrillar collagen most closely mimics the situation in vivo, in which cells in the media have a low proliferative index. Cadmium toxicity of medial smooth muscle cells could be an early event in smoking-induced vascular damage.

In summary, smoking is associated with cadmium deposition in the aortic wall, but there is no selective increase for patients with AAA. The similar tissue concentrations of cadmium in lungs and aortic media of heavy smokers point to
the role of similar mechanisms in the destruction of elastic tissue in lungs and aortas of smokers. Such common mechanisms may explain why lung function is such an important prognostic index for patients with AAA.21 In smokers, the level of cadmium accumulation appears to be sufficient to adversely alter smooth muscle cell metabolism. Other changes in the AAA wall, particularly proteolytic degradation of the elastic connective tissue, could enhance the susceptibility of the aorta to cadmium toxicity, but there is no proof that such mechanisms explain the association between smoking and AAA expansion or rupture.

Acknowledgments
This research was supported by the British Heart Foundation and the Arthur Makey Research Fund.

References
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doi: 10.1161/01.ATV.21.5.863
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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