Prolonged systemic inflammation and damage to the vascular endothelium following intratracheal instillation of air pollution nanoparticles in rats

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KEYWORDS: particles, cardiovascular disease, inflammation, endothelial damage and leucocyte.

ABSTRACT.

BACKGROUND: Exposure to air pollution is associated with cardiovascular disease, including increased morbidity and mortality rates.

OBJECTIVE: The aim of this investigation was to assess the effect, in rats, of intratracheal instillation of particulate air pollution on biomarkers of leucocyte activation and vascular endothelial damage.

METHODS: Air pollution particles (PM10) were instilled into rats, and blood samples were taken three days and six weeks post instillation. Plasma neutrophil elastase and VonWillebrand factor were measured by ELISA.

RESULTS: Plasma neutrophil elastase increased from 175±44 ng/ml at baseline to 288±26 ng/ml 3 days post instillation (p=0.038).   vWF increased from 0.160±0.015 IU/ml at baseline to 0.224±0.015 IU/ml at 3 days post and 0.208±0.01 IU/ml at 6 weeks post (p=0.006, ANOVA). sICAM-1 increased from 17.75±0.70 ng/ml at baseline to 19.03±0.33 ng/ml at 3 days post and 21.72±1.16 ng/ml at 6 weeks post (p=0.009, ANOVA).

CONCLUSION: Instillation caused prolonged systemic inflammation, activation of blood leucocytes and damage to the vascular endothelium.

INTRODUCTION

In epidemiological studies in humans, increased exposure to airborne particulate matter with an aerodynamic diameter of less than 10 microns (PM_{10}) has been implicated in increasing asthma symptoms, cardiovascular malfunction, hospital admissions and morbidity and mortality rates [1,2,3,4]. It has recently been reported that exposure to traffic pollution by residence near major roads is associated with a decrease in ankle-brachial index and an increased incidence of peripheral arterial disease [5,6]. Acute exposure to particulate air pollution, fine particles in particular, is associated with increased mortality [7].

The mechanisms by which exposure increases risk is poorly understood [8]. Our research group reported an increase in plasma viscosity in rats which correlates with the degree of inflammatory changes in the lung after instillation of nanoparticles [9]. Acute cardiopulmonary alterations are associated with exposure to fine particulates in rats [10,11]. Acute changes in blood levels of biomarkers of inflammation have been reported after short term exposure to diesel exhaust in humans [12,13,14], to PM2.5 in rats [15] and after intrapharyngeal instillation of particles in atherosclerotic rabbits [16]. Exposure to residential heating emissions results in changes in platelet biochemisty [17]. Studies have generally focused on short term acute inflammatory effects but any long term inflammatory effects will have an important impact on health outcomes after exposure to even short episodes of poor air quality.

Atherosclerosis is an inflammatory disease of the arteries, and inflammatory changes to leucocytes and damage to the vascular endothelium are associated with

atherosclerosis. Inflammation causes activation of leucocytes which is associated with actin polymerisation [18], and increased actin polymerisation has previously been shown in rat monocytes after *in vivo* exposure to air pollution particles [19]; leucocyte activation is known to lead to decreased flow [19, 20, 21, 22]. Activated leucocytes release other inflammatory mediators such as proteolytic enzymes and free radicals [23, 24, 25]. These released inflammatory mediators may cause activation of and damage to the vascular endothelium; an initial step in the development of atherosclerosis [24]. Damage to the vascular endothelium is associated with an increase in blood vWF and sICAM-1 concentration and increases in these biomarkers are independent risk factors for cardiovascular disease [26, 27, 28, 15]. We have previously reported an association between leucocyte activation, release of the proteolytic enzyme, neutrophil elastase, and endothelial damage in peripheral vascular disease [29], in bone marrow transplantation [30] and in angioplasty [31].

Inflammatory changes associated with damage to the vascular endothelium could, therefore, be the cause of the association between particle exposure and cardiovascular disease. The aim of the study described here was to test the hypothesis that exposure to airborne particulate pollution causes inflammatory activation of blood leucocytes which leads to damage to the vascular endothelium. The aim was also to determine the longer term effects of exposure. To this end the effect, in rats, of intratrachaeal instillation of PM10 on biomarkers of leucocyte and endothelial function was measured. The effect of a single instillation was measured for an extended period of six weeks.

MATERIALS AND METHODS

Collection and preparation of PM10

Urban air pollution particles were collected using a high volume impactor (HVI). The machine is capable of collecting large amounts of sample within a foam substrate. The particles are easily removed from the substrate with minimum sample alteration. The HVI runs on a 40-Amp electrical supply, sucking air at 1100 l/min through the head containing the foam substrates. There are cylindrical inlets through which the air passes. Air passes through a debris collector, where any particles bigger than 10 μm are trapped on a polyurethane foam (PUF) substrate. Particles smaller than 10 μm pass through a second cylindrical inlet and impact in a second PUF substrate. The PUF were collected and chopped into small pieces then vigorously agitated with distilled water. The particles were then freeze dried and stored at 4C [32]. A known mass of particles was resuspended in 0.15M saline and 'wetted' (suspended in solution) by 15 min sonication. Particle physicochemistry was assessed by transmission electron microscopy, x-ray microanalysis, and quantitative image analysis (Table 1). Particles consisted of about 10% ultrafine particles (<100nm).

Animals and experimental protocol

All animals were treated humanely under guidelines provided by Cardiff University and the local ethical committee. Study approval was obtained from the local ethical committee and by a Home Office animal project licence. The animal and experimental protocols used were as previously reported [9]. Briefly, male Sprague Dawley rats (200 g, n=10 per group) were purchased from Charles River (UK) and were acclimatised within the animal holding facility for one week prior to instillation. The animals were kept on wire-bottom cages with pelleted food and tap water *ad libitum*. The animals were lightly anaesthetised with Halothane before receiving

intratracheal instillations. Controls were instilled with saline only whilst test animals were instilled with saline containing 2.5mg/kg of particles. Animals were harvested 3 days and 6 weeks after instillation. There were no difference in control animal biomarkers between three days and six weeks post instillation.

Blood samples and cell counting

Blood samples were collected, by cardiac puncture, into sufficient tri-potassium EDTA to give a final concentration of 1.5 mg/ml. Haematological parameters were determined by performing a blood count using Serono-Baker System 9000 automated cell counter (Bio-Stat Diagnostic Systems, Stockport, Cheshire, UK). Within four hours of collection samples were centrifuged at 1500g for 10 minutes (Sanyo Harrier 18/80) and plasma was removed and stored at -80°C (Sanyo VIP series) until analysis.

Measurement of plasma concentration of soluble biomarkers of inflammation.

Plasma α-1-trypsin neutrophil elastase inhibitor complex was measured by ELISA using sheep anti-human neutrophil elastase and peroxidise-conjugated sheep anti-human α-1-antitrypsin (The Binding Site, Birmingham, UK) and PMN leucocyte elastase calibrator (Merck Ltd, UK). vWf was measured by ELISA using polyclonal anti-von Willebrand Factor primary antibody (Dako,UK) and HRP conjugated polyclonal anti-von willebrand Factor secondary antibody (Dako,UK) and vWF standard (NIBSC,UK). Rat sICAM-1 was measured by commercially available ELISA (R&D systems, UK).

Statisical Analysis

All statistical analysis was performed using Minitab 12 software (Minitab Ltd, Coventry, UK) Values are quoted as mean ± standard error (SEM). Differences between groups were analysed by one-way analysis of variance (ANOVA) where appropriate. Correlation analysis between variables was performed by calculation of Pearson correlation coefficient and corresponding P values were calculated.

RESULTS

Instillation of particulates into the lung of rats resulted in changes in biomarkers of inflammation in the systemic blood circulation. The changes continued for at least six weeks after the instillation of particles.

Plasma neutrophil elastase increased from 175±44 ng/ml (mean±SEM) at baseline to 288±26ng/ml 3 days post instillation with p=0.038 when determined by two sample T-test. At 6 weeks post instillation the plasma neutrophil elastase remained elevated at 292±51ng/ml. The increase in elastase over the extended time period was on the border of significance with p=0.06 when determined by ANOVA. Results are summarised in Figure 1.

There was no significant change in total leucocyte count in animals during the experiment. Changes in neutrophil elastase are, therefore, not simply a reflection of changes in the total number of leucocytes (Table 2). Neutrophil elastase is secreted from normal neutrophils and the amount of enzyme secreted tends to increase with activation of neutrophils. As neutrophil elastase is secreted form normal neutrophils

and increase in concentration of plasma elastase could occur simply because the total number of neutrophils in blood has increased. As no such increase in cell count occurs during the experiment then the increase in plasma concentration of neutrophil elastase recorded here results from increased secretion from neutrophils.

There was a statistically significant increase in the two biomarkers of endothelial damage. vWF increased substantially from 0.160 ± 0.015 IU/ml (mean±sem) at baseline to 0.224 ± 0.015 IU/ml at 3 days post instillation. Plasma vWF concentration remained elevated, at 0.208 ± 0.01 IU/ml for at least 6 weeks post instillation (p=0.006, ANOVA). A single episode of exposure, therefore, resulted in a prolonged increase in plasma concentration of vWF. vWF increased soon after exposure and decreased slightly towards baseline but at six weeks post instillation was still elevated (Figure 2).

sICAM-1 increased from 17.75 ± 0.70 ng/ml (mean±sem) at baseline to 19.03 ± 0.33 ng/ml at 3 days post and 21.72 ± 1.16 ng/ml at 6 weeks post (p=0.009, ANOVA). Again a single instillation of particles resulted in a prolonged increase in the plasma concentration of sICAM-1. sICAM-1 concentration increased soon after exposure and was still elevatated, and perhaps continued to increase, at six weeks post instillation (Figure 3).

There was a weak but significant correlation between plasma neutrophil elastase concentration and vWF concentration with a Pearson correlation coefficient of 0.329 and p=0.047 (Figure 4).

DISCUSSION

The increase in neutrophil elastase observed here is an indication that instillation of air pollution particles into the lung of rats causes activation of leucocytes in systemic blood. Neutrophil elastase is a granule serine proteinase which is capable of degrading almost all extracellular matrix proteins as well as variety of key plasma proteins. It is released in response to neutrophil activation by inflammatory stimuli or phagocytosis and it also induces the release of pro-inflammatory cytokines such as interleukin-6 and 8. Neutrophil elastase is, therefore, a useful biomarker of leucocyte activation. Usually the activity of neutrophil elastase released to extracellular region is strictly regulated by endogenous macromolecular inhibitors, however in some inflammatory cases where large numbers of neutrophils are activated a large amount of elastase is released and the inhibitors are inactivated by neutrophil oxidative products, this results in severe tissue damage. For example neutrophil elastase may play an important role in the pathophysiology of acute lung injury and lung inflammation [33, 34].

The concentration of neutrophil elastase in plasma may vary with the number of leucocytes in blood; if the number of leucocytes in blood is higher, then the total amount of secreted enzyme is likely to be higher. In the experiments described here there is no change in the total leucocyte count so the measured changes in neutrophil elastase reflect an increase in secretion of enzyme from the leucocytes.

Interestingly, there is a statistically significant change in the red blood cell haematological parameters. At three days post instillation there is a decrease in total

red cell count which then rises again, to slightly above control, at six weeks post instillation. The decrease in red cell count at 3 days could be caused by haemolysis in the blood circulation during the initial stages post instillation. Particle associated metals have been found in the plasma four and twenty four hours following lung instillation [35]. These translocated metals could cause haemolysis. There is a slight increase in red cell count, and other red cell indices such as haematocrit and haemoglobin concentration at six weeks post instillation. This increase could be caused by a decrease in lung function caused by lung damage resulting from particle instillation. An increase in red cell count is an independent risk factor for cardiovascular disease [36]. This effect on red cell count could be another mechanism by which exposure to particulate air pollution could increase cardiovascular risk.

The particle associated increase in plasma neutophil elastase, reported here, is associated with damage to the vascular endothelium. Von Willebrand factor [29,31] and sICAM-1 [26] levels are biomarkers of endothelial damage and increased levels are associated with a significant increase the risk of cardiovascular disease. The relationship between plasma neutrophil elastase concentration and damage to the vascular endothelium reported here is similar to that we have previously reported in peripheral vascular disease [29], bone marrow transplantation [30] and angioplasty [31]. Neutrophil elastase causes damage to the vascular endothelium as it is a potent serine protease [37]. Once it's released it cleaves elastin, collagen, fibronectin and proteoglycans; it also exposes recognition sites that bind cellular integrin and tyrosine receptors allowing overlapping of the cell response to tissue injury. The products of

elastin degradation are recognized by elastin receptors and proatherogenic effects involving monocytes and vascular smooth muscle cells are activated [38].

The increased level of soluble ICAM-1 reported here not only reflects damage to the endothelium but also indicates that there is increased expression of ICAM-1 on the surface of the endothelium. This increase ICAM-1 on the endothelium will allow the activated neutrophils to adhere to the endothelium. When the vascular endothelium is activated, expression of adhesion molecules increases and leucocytes start rolling on the endothelium with the help of selectins. Integrins, such as ICAM-1 are activated by proteolytic attack, chemokines, lipid mediators and other pro-inflammatory molecules presented on the surface of the endothelium. Once activated the ICAM-1 mediates adhesion of the leucocytes which migrate across the endothelium and enter into inflamed tissue.

Adhesion of neutrophils will increase the damage they can cause to the endothelium. When adherent neutrophils release proteolytic enzymes and other damaging intermediates, such as oxidative radicals, the close association between the neutrophil and the endothelium results in a high concentration of damaging molecules close to the endothelium and the potential for damage increases. Furthermore, damaging molecules may even be highly concentrated in the small gap between an adherent neutrophil and the vascular endothelium. At this higher concentration the level of proteolytic enzyme will be greater than the inhibitory capacity of the α-1 plasma inhibitor. The potential for endothelial damage is then even further increased.

Adhered neutrophils can extravasate and migrate into the surrounding tissues. These neutrophils may then take with them any particles which may have been phagocytosed. This could be a mechanism of entry of particles into tissues distant from the site of exposure. Movement of particles around the body has important implications for the potential toxicity of particles as translocated particles could accumulate in tissues and become involved in a range of unpredictable toxicological processes. Inhaled particles have been found in the systemic circulation [39] and in the brain [40, 41]. The mechanisms involved in translocation of particles and the potential toxicity associated with this translocation is currently poorly understood.

Interestingly the inflammatory changes reported her persist for weeks after the initial instillation of air pollution particles. The single dose of particles, used here, resulted in inflammatory changes which were still measurable six weeks after the dose. Previous studies on the inflammatory effect of air pollution have focused either on the effects of long term exposure or on the short term acute effects of exposure. Acute exposure studies have focused on effect in the hours or days after exposure. The results presented here indicate that even short term episodes of high levels of particulate air pollution could cause systemic inflammatory changes which increase the risk of developing cardiovascular disease.

It has recently been reported that striking similarities exist between the dose-response relationship between cardiovascular mortality and exposure to particulate air pollution and cigarette smoke [42]. For both cigarette smoke and air pollution the increase in risk is relatively steep at low exposure and flattens out at higher exposure. The authors suggest that this has important public health implications; exposure to

even relatively low levels of particulate air pollution could present a significant health risk.

Here we report that the time scale for inflammatory changes following exposure to particulate air pollution may be similar to those reported for exposure to tobacco smoke. Markers of inflammation in smokers remain elevated for many months after smoking cessation [43] and, levels of inflammatory markers improve within 5 years of smoking cessation but take over 20 years to revert to levels of those who have never smoked [44]. The fact that even low doses of particulate air pollution could be associated with increased cardiovascular disease and that inflammation persists long after a short episode of exposure has important implications for public health. Exposure to even short episodes of poor air quality could be associated with long term increases in cardiovascular risk.

In summary, a single instillation of air pollution particles results in activation of leucocytes and release of proteolytic enzymes which damage the vascular endothelium. Activation of the endothelium increases adhesion of leucocytes and migration of cells, possibly loaded with toxic particles, into tissues which are distant from the lung. These inflammatory changes persist long after the initial exposure event. Exposure to even short episodes of poor air quality could significantly increase cardiovascular risk.

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Table 1. Size distribution and inorganic elemental composition of particles.

Diameter (nm)	% Ultrafine (<100nm)	%Fine (0.1-2.5μm)	%Coarse (>2.5μm)	Elemental composition (inorganic)
30	10.1	89.5	0.4	C,O,Na,Mg,Al,Si,P,S,Cl,K,Ca, Ti,Mn,Cr, Fe,Zn,Pb

Table 2. The effect of intratraceal instillation of particles on haematological parameters. Values expressed as mean(± SD). P determined by ANOVA. [WBC, total leucocyte count; RBC, total red cell count; HGB, haemoglobin concentration; HCT, haematocrit; PLT, total platelet count].

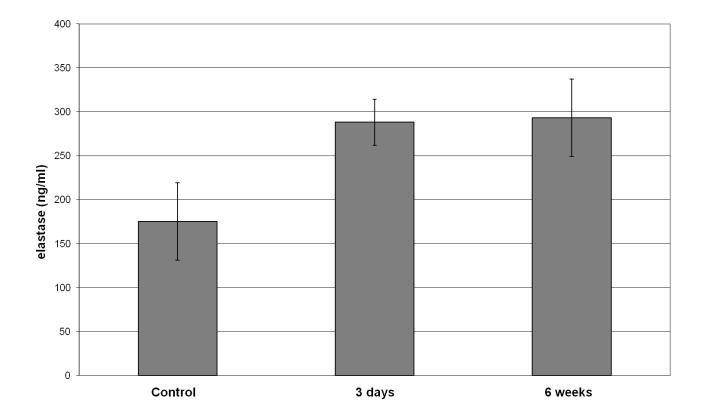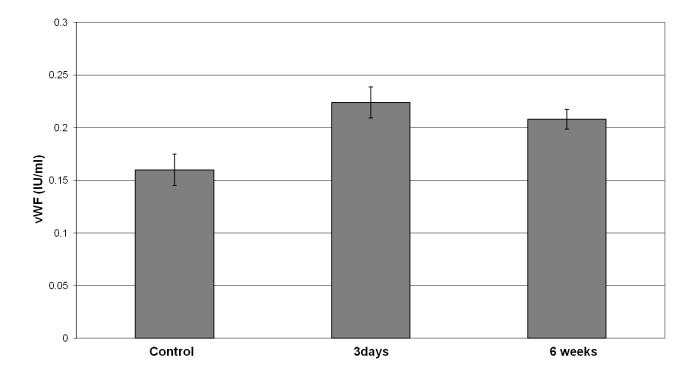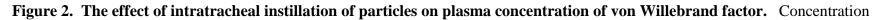|	*control* *(n=10)*	*3 days post* *(n=15)*	*6 weeks post* *(n=15)*	*p*
WBC (x 10^9/L)	6.53(1.36)	6.03(1.48)	5.72(1.48)	0.478
RBC (x 10^{12}/L)	7.17(1.17)	6.09(0.53)	8.05(0.46)	≤0.001
HGB (g/dL)	13.98(1.04)	13.22(0.69)	14.10(0.455)	0.032
HCT(%)	41.7(5.14)	38.4(3.12)	43.8(2.39)	0.01
PLT (x10^9/L)	1042(129)	1115(158	1046(214)	0.582

Figure 1. The effect of intratracheal instillation of particles on plasma concentration of Plasma α-1-trypsin neutrophil elastase inhibior complex. Concentration determined by ELISA in controls and at three days and six weeks post intratracheal instillation of particles. Results expressed as mean (±SEM); n= 10 for control animals and 15 for test animals. P, determined by ANOVA = 0.06.

Figure 2. The effect of intratracheal instillation of particles on plasma concentration of von Willebrand factor. Concentration determined by ELISA in controls and at three days and six weeks post intratracheal instillation of particles. Results expressed as mean (±SEM); n= 10 for control animals and 15 for test animals. P, determined by ANOVA = 0.006.
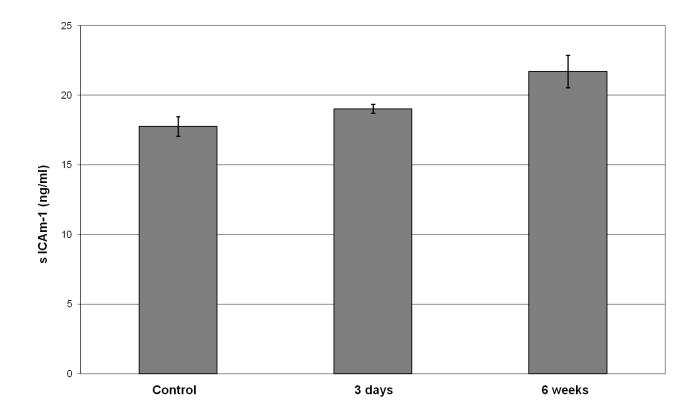
Figure 3. The effect of intratracheal instillation of particles on plasma concentration of soluble ICAM-1. Concentration determined by

ELISA in controls and at three days and six weeks post intratracheal instillation of particles. Results expressed as mean (±SEM); n= 10 for

control animals and 15 for test animals. P, determined by ANOVA = 0.009.
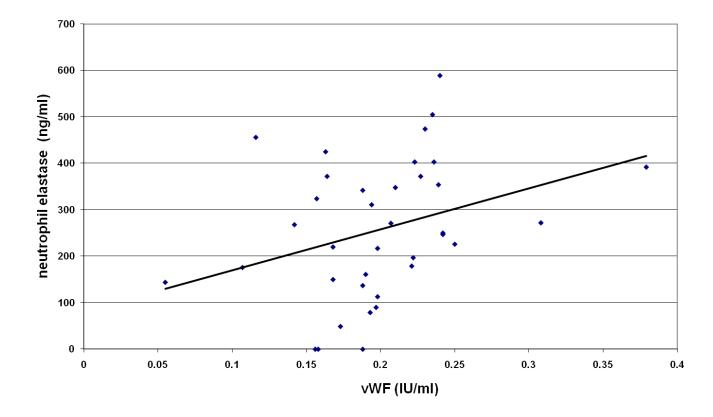
Figure 4. The relationship between plasma α-1-trypsin neutrophil elastase inhibior complex concentration and von Willebrand factor concentration. Analysis by Pearson correlation r = 0.33 and p = 0.047.

Table and Figure Legends

Table 1. Size distribution and inorganic elemental composition of particles.

Table 2. The effect of intratraceal particle instillation on haematological parameters. Values expressed as mean(± SD). P determined by ANOVA. [WBC, total leucocyte count; RBC, total red cell count; HGB, haemoglobin concentration; PLT, total platelet count].

Figure 1. The effect of intratracheal instillation of particles on plasma concentration of plasma α-1-trypsin neutrophil elastase inhibior complex. Concentration determined by ELISA in controls and at three days and six weeks post intratracheal instillation of particles. Results expressed as mean (±SEM); n= 10 for control animals and 15 for test animals. P, determined by ANOVA = 0.06.

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Figure 3. The effect of intratracheal instillation of particles on plasma concentration of soluble ICAM-1. Concentration determined by ELISA in controls and at three days and six weeks post intratracheal instillation of particles. Results expressed as mean (±SEM); n= 10 for control animals and 15 for test animals. P, determined by ANOVA = 0.009.

Figure 4. The relationship between plasma α-1-trypsin neutrophil elastase inhibior complex concentration and von Willebrand factor concentration. Analysis by Pearson correlation r = 0.33 and p = 0.047.