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Changes in atherosclerotic plaques induced by inhalation of diesel exhaust

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ABSTRACT

Objective: Exposure to particulate matter air pollution may be an independent risk factor for cardiovascular morbidity and mortality; however, the biological mechanisms are unclear. We hypothesize that exposure to diesel exhaust (DE), an important source of traffic-related particulate air pollution, promotes changes of atherosclerotic plaque component that may lead to plaque vulnerability.
Methods and results: 30-week old ApoE knockout mice fed with regular chow inhaled DE (at 200 $\mu g/m^3$ of particulate) or filtered-air (control) for 7 weeks (6 h/day, 5 days/week) (12 mice/group). Total number of alveolar macrophages ($p < 0.01$) and alveolar macrophages positive for particles ($p < 0.0001$) were more than 8-fold higher after DE inhalation than the control. DE inhalation caused 1.5 to 3-fold increases in plaque lipid content ($p < 0.02$), cellularity ($p < 0.02$), foam cell formation ($p < 0.04$), and smooth muscle cell content ($p < 0.05$). The expression of oxidative stress markers, iNOS, CD36, and nitrotyrosine was significantly increased by 1.5 to 2-fold in plaques, with enhanced systemic lipid and DNA oxidation ($p < 0.02$). Increased foam cells and the expression of iNOS ($R^2 = 0.72$, $p = 0.0081$) and CD36 ($R^2 = 0.49$, $p = 0.015$) in plaques were positively correlated with the magnitude of DE exposure.
Conclusions: Exposure to DE promotes changes in atherosclerotic plaques characteristic of unstable vulnerable plaques. Increased systemic and plaque oxidative stress markers suggest that these changes in plaques could be due to DE-induced oxidative stress.

1. Introduction

Epidemiological studies conducted over the past 20 years showed that exposure to ambient particulate matter air pollution with aerodynamic diameter less than 2.5 μm ($PM_{2.5}$) may be an independent risk factor for increased cardiovascular morbidity and mortality [1,2], and recent studies suggest that reducing ambient particles resulted in declined cardiovascular morbidity and deaths [3,4]. Evidence from other and our own laboratories suggested that deposition of particles in the lung causes alveolar inflammation resulting in a systemic inflammatory response that impacts the blood vessels [5–8], which could be responsible for the downstream adverse cardiovascular effects [1]. This hypothesis is supported by several animal studies [6–8] and human studies showing that elevations of 10 $\mu g/m^3$ and 20 $\mu g/m^3$ in $PM_{2.5}$ were associated with 5.9% and 12.1% increases in the development of atherosclerosis, respectively [9].

Inflammation plays a central role in all stages of atherosclerosis development [10]. Exposure to particulate matter air pollution results in excessive production of reactive oxidative species (ROS) [11]. These ROS contribute to many cardiovascular complications via exacerbating interactions with lipids, proteins, and DNA. An in vitro study showed that diesel exhaust particle (DEP)-induced ROS exerted lipid peroxidation to produce oxidized low-density-lipoprotein (oxLDL), which can serve as a stimulus for monocyte migration into the sub-endothelial space representing an key step in atherogenesis [10,12]. These monocyte-derived macrophages phagocytose oxLDL, contributing to foam cell formation, and the development of fatty streaks, a hallmark of early atherosclerotic lesion [10]. Macrophage CD36 is the major scavenger receptor for oxLDL, hence promoting the progression of atherosclerosis [13]. Active unstable atherosclerotic plaques are characterized by

Abbreviations: DE, diesel exhaust; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; H and E, hematoxylin and eosin; HPRT1, hypoxanthine phosphoribosyltransferase-1; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide synthase; IL-1, interleukin-1; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; NF-κB, nuclear factor-kappa B; NT, nitrotyrosine; OxLDL, oxidized low-density-lipoprotein; $PM_{2.5}$, particulate matter air pollution with aerodynamic diameter less than 2.5 μm; RT-PCR, reverse transcription polymerase chain reaction; ROS, reactive oxidative species; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; 8-OH-dG, 8-hydroxy-2-Deoxy guanosine; 15-F_{2t}-IsoP, 15-F2t-isoprostane.

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increased lipid accumulation, foam cell formation, smooth muscle migration from the sub-endothelial layer and a thin fibrous cap [14]. Plaque disruption and atherothrombosis are the underlying causes of approximately 60–80% of all sudden cardiac deaths [15].

Diesel exhaust particulate is a major component of urban $PM_{2.5}$, accounting for up to 90% of the fine particulate mass in ambient air of many major cities, such as London [16]. Diesel Exhaust (DE) is a mixture of fine particles and gases, and represents a useful model of traffic-related air pollutants. The particulate from DE consists of a central carbon core nucleus onto which an estimated 18,000 combustion products are absorbed, including organic chemicals, such as polycyclic aromatic hydrocarbons, and transition metals [17,18].

Recent studies have shown an association between the progression of atherosclerosis and people living near major roads, and traffic-related air pollution exposure and acute coronary events [19]. The association between DE exposure and cardiovascular diseases is compelling; however, the impact of DE exposure on atherogenesis is poorly understood. In this study we exposed ApoE knockout mice to DE via whole-body inhalation for 7 weeks, and measured morphological alteration of atherosclerotic lesion to test the hypothesis that DE exposure causes changes in atherosclerotic plaque characteristic of unstable plaque or plaque vulnerable to cause acute vascular events. We also explored the impact of the magnitude of DE exposure and downstream oxidative stress on the changes in plaque morphology.

2. Material and methods

2.1. Exposure protocol and experimental animals

Characteristics of the exposure system have been described elsewhere in detail [20] (See online supplementary "Material and methods" for more details).

Male ApoE knockout mice fed with regular chow, at the age of 30-week, were exposed for 7 weeks (5 days/week, 6 h/day) to DE controlled at the concentration of 200 $\mu g/m^3$ $PM_{2.5}$. Mice exposed to filtered air were the control (12mice/group). Animal procedures were approved by the Animal Care and Use Committee of the University of Washington.

2.2. Sample processing and image acquisition

After exposure, sodium pentobarbital (100 mg/kg, Abbott Laboratories) and heparin sulfate (500 U/kg) were administered intraperitoneally. Plasma, thoracic aorta, aortic root, lung and urine were collected and kept in appropriate conditions until assay. For morphometric analysis, images were captured by a spot digital camera (Microspot, Nikon), coded and analyzed using Image Pro Plus software. 10–12 fields per view of each animal were randomly chosen.

2.3. Plasma cholesterol and triglyceride

Plasma cholesterol and triglyceride levels were measured using commercially available kits (Cayman Chemical).

2.4. Lung tissue analysis

Lungs were inflated and fixed with 10% neutral formalin for 24 h, cut into 4 pieces longitudinal slices, embedded in paraffin blocks, sectioned at 10 μm and stained with hematoxylin and eosin (H and E). Random images were captured to represent the whole lung and analyzed using point counting to determine the volume fraction (V/v%) of alveolar macrophages. The V/v% of alveolar macrophages positive for particulate matter was measured as an indicator for the magnitude of lung exposure.

2.5. Histo- and immunohistochemical analysis of atherosclerotic plaque

Aortic root was used to quantify the changes in plaque. Aortic root tissues were fixed with 10% neutral formalin for 24 h, embedded in paraffin, sectioned at 5 μm and stained for Movat pentachrome. Point counting was used to determine the V/v% of plaques, total cell counts (plaque cellularity) and foam cells. The nuclei of cells in each lesion were enumerated and normalized to the plaque size.

Picro-sirius red (Sigma) staining was used to identify collagen content and collagen was quantified (V/v%) in plaque tissues.

The expression of iNOS, CD36, nitrotyrosine and α-actin was quantified after immunohistochemical staining for aortic root tissues. Sections were incubated with antibodies F4/80, a maker for mature macrophages (1:50, AbD Serotec); inducible nitric oxide synthase (iNOS) (1:100); CD36 (1:50); nitrotyrosine (NT) (1:400), and α-actin (1:600) at 4 °C overnight. Subsequently, sections were incubated with biotinylated goat anti rabbit IgG (1:800, Vector Laboratories), followed by avidin- biotin conjugated alkaline phosphatase and Vector red (Vector Laboratories) to detect the antigen-antibody complexes. The area of positive staining was recognized, and the volume fraction (V/v%) of iNOS, CD36 and NT was determined.

2.6. Lipids in plaque

Frozen aortic root was embedded in OCT (Sakura Finetek), sectioned at 5 μm, stained with Oil-Red-O and V/v% of positive staining was determined.

2.7. Systemic oxidative stress

Urine samples were purified using 8-isoprostane affinity sorbent, and 15-F_{2t}-isoprostane (15-F_{2t}-IsoP), a marker of lipid oxidation, was measured using a 8-Isoprostane EIA kit (Cayman Chemical). As a marker of DNA oxidation, 8-hydroxy-2-deoxy guanosine (8-OH-dG) concentration was measured using a 8-OH-dG kit (Cayman Chemical). Creatinine levels were measured by a creatinine assay kit (Cayman Chemical). The plasma myeloperoxidase (MPO) levels were also measured using an ELISA kit (Invitrogen).

2.8. Statistical analysis

Data are shown as mean ± SEM. The statistical significance was evaluated using the unpaired Student's *t*-test for simple comparison between two values. Linear regression modeling was used to assess the relationship between exposure markers (alveolar macrophages with particles) and changes in plaques and oxidative stress markers. In all experiments, *n* equals the number of mice from which samples were obtained. $p < 0.05$ was considered to be significant.

3. Results

3.1. Alveolar macrophages in lung tissue

DE exposure significantly increased the number of alveolar macrophages positive for particles (3.5 ± 1.2% in Filtered air vs. 85.7 ± 1.7% in DE; $p < 0.0001$) (Fig. 1A). The total number of alveolar macrophages was also increased in DE exposure group, compared