Nano-Sized Carbon Black Exposure Exacerbates Atherosclerosis in LDL-Receptor Knockout Mice

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Background Associations between exposure to particulate matter and susceptibility to cardiovascular events have been reported. Although the underlying mechanisms are not fully understood, this association seems to be particularly exaggerated in the presence of atherothrombotic risk factors. The present study was undertaken in low-density lipoprotein receptor knockout (LDLR/KO) mice to test the hypothesis that long-term exposure to a high dose of nano-sized carbon black (CB) exacerbates atherosclerotic lesions.

Methods and Results LDLR/KO mice were subjected to a 10-week intratracheal dispersion of CB (1 mg/week) or air under a 0% or 0.51% cholesterol (Chol) diet. Development of aortic lipid-rich lesions was detected in mice under a 0.51% Chol diet with or without CB dispersion, but not in mice fed a 0% Chol diet with or without CB. Quantification of the area stained with oil red O revealed the highest percentage in CB-treated mice on a 0.51% Chol diet among the 4 groups. One-way ANOVA indicated CB-treated mice with 0.51% Chol diet had a significantly higher percentage of positive staining than vehicle-treated mice with 0.51% Chol diet (p<0.05).

Conclusions In LDLR-deficient mice under a high Chol diet, exposure to CB resulted in acceleration of development of atherosclerosis. (Circ J 2007; 71: 1157–1161)

Key Words: Atherosclerosis; Epidemiology; Risk factors

Exposure to particulate matter has been recognized as a risk factor for atherothrombotic diseases. We have previously shown that both carbon black (CB) and water-soluble fullerene (C60(OH)24) induce cytotoxic injury, inhibit cell growth and upregulate pro-inflammatory genes such as ICAM-1, E-selectin and CCL2 in human umbilical vein endothelial cells (HUVEC), suggesting a possible atherothrombogenic role of nano-sized particulate matter in the development of air-pollution-mediated cardiovascular disease.

Interestingly, in cultured macrophage cells, cytotoxicity of either CB or C60(OH)24 was markedly enhanced by co-treatment with oxidized-low-density lipoprotein (LDL), leading to the development of lipid-laden macrophages and secretion of pro-matrix metalloproteinase 9 (pro-MMP9). Therefore, it can be speculated that CB may exacerbate atherosclerosis in the presence of risk factors for cardiovascular disease. In the present study, we tested the hypothesis that exposure to CB may exacerbate atherosclerotic lesions in a model using LDL receptor (LDLR) knockout (LDLR/KO) mice fed a high cholesterol (Chol) diet.

Animal Model
Male LDLR/KO mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice homozygous for LDLR-null allele were subsequently generated and kept in a temperature-controlled facility on a 12-h light–dark cycle with free access to normal chow and water. At 6 weeks of age, the mice were randomly divided into 4 groups (5 per group). Two groups of mice were subjected to a 10-week endotracheal dispersion of autoclaved CB (1 mg per animal/week) and given isonergy diets containing 0% or 0.51% Chol. Another 2 groups of mice underwent intratracheal dispersion of air only under isonergy diets with 0% or 0.51% Chol. Intratracheal dispersion of CB and air was performed once a week for 10 weeks under light ether anesthesia using the DP-4 dry powder insufflator (Penn-Century Inc, Philadelphia, PA, USA). CB (The Association of Powder Process Industry and Engineering, Japan) was autoclaved before administration. The distribution of particle size (nm) is presented in Fig 1. At the end of the experiment, the mice were anesthetized by pentobarbital, and then the blood was collected directly from the abdominal aorta. The blood samples were stored at –80°C until analysis.

The acute effect of CB dispersion on circulating levels of C-reactive protein (CRP) was investigated in a separate group of male LDLR/KO mice aged 10–14 weeks. Mice were first fed with a 0.51% Chol diet for 3 days and were subjected to single administration of CB (1 mg per animal) or air using the identical protocol as described above (5 mice each in CB-treated and air-treated groups). Blood samples were collected 24 h after dispersion with CB or air.

All animal experiments were performed in accordance with the institutional ethical guidelines for experiments with animals.

Histochromic Analysis
The extent of atherosclerosis was assessed in the longitu-
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Finally opened aortas that were stained with oil red O and pinned flat on a dish. The stained aortas were examined under a stereomicroscope (Olympus, model SZX12, Tokyo, Japan) connected to a RGD camera (Olympus, model DP12). Captured images were analyzed with Adobe Photoshop CS2 to quantify the total surface area of the aorta and the positively stained area, using a square grid as a guide. The positively stained lesion looked brighter in the images than the area not stained with oil red O (Figs 2A, B). The percentage of the stained area was determined by the number of the

Fig 1. Distribution of carbon black particles (nm). Particle diameter was measured by a Particle Size Analyzer (UPA-EX150, Nikkiso, Japan). Mean size was 120.7 nm (mode = 111.4 nm, geometric SD = 1.68).

Fig 2. Oil red O staining of the plaque area of aortic tissue samples from low-density lipoprotein receptor knockout (LDLR/KO) mice. The extent of atherosclerosis was assessed in aortas that were opened longitudinally. Representative aortic tissues from LDLR/KO mice on a 0% cholesterol (Chol) diet with or without carbon black (CB) treatment (A) and for those on a 0.51% Chol diet with or without CB treatment (B). Development of lipid-rich aortic lesions is more evident in the mice on the 0.51% Chol diet than in those on the 0% Chol diet.
Carbon Black Exposure in LDLR/KO Mice

Results

The LDLR/KO mice underwent intratracheal dispersion of CB (1 mg per animal) once a week for a total of 10 weeks. Although no difference in body weight between the 4 groups was observed at baseline, and all mice experienced an increase in body weight with advancing age, the mice treated with CB tended to be smaller than those treated with vehicle (Table 1). No significant differences were observed in Chol and triglyceride levels among the 4 groups (data not shown).

Development of aortic lipid-rich lesions occurred in mice under a 0.51% Chol diet with or without CB infusion, but not in the mice fed a 0% Chol diet with or without CB (Figs 2A, B). Quantification of the area stained with oil red O revealed the highest percentage in CB-treated mice on a 0.51% Chol diet among the 4 groups (p<0.0001 by ANOVA). One-way ANOVA indicated that there were significant differences among the 4 groups (p<0.0001). Subsequent comparison revealed CB-treated mice under the 0.51% Chol diet had a significantly higher percentage of positive staining than vehicle-treated mice with the same diet (p<0.05, Fig 3).

The effect of a single CB dispersion on circulating levels of CRP was examined in the separate group of mice. Circulating levels of CRP measured 24 h after the single dispersion of CB or air were significantly higher in mice exposed to CB than in mice treated with air (Fig 4, p<0.0005), indicating an acute inflammatory response.

Although the presence of CB in pulmonary macrophage-like cells in CB-treated mice under a 0.51% Chol diet was confirmed, CB was not detected by electron microscopic examination of the aortas, livers, kidneys and spleens (data not shown).

Discussion

Our study demonstrates that intratracheal dispersion of CB can induce the development of atherosclerosis in LDLR/KO mice fed a diet with 0.51% Chol. It has previously been shown that 6-month exposure to low concen-
tations of ambient particles of less than 2.5 \( \mu \text{m} \) induced acceleration of atherosclerosis in apolipoprotein E-deficient mice under a high-fat diet. Similarly, exposure of particulate matter (<10 \( \mu \text{m} \)) for 4 weeks by intratracheal instillation exacerbated the atherosclerotic lesions in the coronary arteries and aortas of rabbits susceptible to the development of atherosclerosis. Our results are in accordance with these findings, despite differences in dosages, length of the experimental period, particle size, animal model and the method of exposure.

Although the exact mechanisms are not yet determined, a study by Nemmar et al has demonstrated the rapid translocation of inhaled nano-sized carbon particles into the bloodstream in humans. CB dispersed into trachea might be translocated into bloodstream and then act as an atherothrombogenic agent on vascular tissues. We have previously shown that nano-sized CB has a direct atherogenic influence on cultured endothelial cells, as indicated by cytotoxicity, inhibition of cell growth and upregulation of pro-inflammatory genes such as ICAM-1, E-selectin and CCL2. In addition, our recent study has demonstrated that nano-sized CB exacerbated the formation and cytotoxicity of foam cells (macrophages) induced by the treatment with oxidized-LDL.

Although the rapid translocation of inhaled \( ^{99} \text{m} \text{Tc} \)-labeled carbon particles into the bloodstream in humans has been reported, a more recent study conducted using the same dose of technegas (100 MBq) has found most of the inhaled carbon nanoparticles remained in the lungs (approximately 95% at 6 h after inhalation). Similar observations have been reported in animal models and humans. We attempted to clarify whether dispersed CB might be translocated into circulating blood and taken up into atherosclerotic plaques. It was evident that dispersed CB had reached the alveolar regions, as evidenced by the existence of macrophage-like cells containing CB (data not shown). However, electron microscopic examination did not reveal the existence of CB in plaque lesions. Although we cannot exclude the possibility that a trace amount of CB might have translocated into the systemic circulation, such a low level of CB appears to be nontoxic. Our in vitro experiments indicated extremely high concentrations of CB were necessary to cause cytotoxicity in endothelial cells and macrophages.

Our observation that CB dispersion induced atherosclerosis in LDLR/KO mice under a 0.51% Chol diet may be partly explained by higher levels of circulating CRP observed in CB-treated than in air-treated mice in response to acute exposure of CB. A local inflammatory response in the lung as a result of accumulation of inhaled particles may lead to endothelial dysfunction and atherothrombogenesis. As originally proposed by Seaton et al it has been hypothesized that ultrafine particles are able to induce oxidative stress and inflammation in the lung in susceptible populations, exerting detrimental effects on the cardiovascular system through the release of pro-inflammatory mediators and coagulation factors. Results from animal and human studies have shown pulmonary inflammation after inhalation of concentrated ambient air particles, as indicated by the increased neutrophils in the bronchoalveolar lavage fluid. A more recent study examining the acute deleterious effects of intratracheal instillation of 6 different ultrafine carbon particles in mice has demonstrated that those with the smallest diameter (7–12 nm) and the largest surface area (807 m²/g) among the 6 types of particles yielded the most pronounced inflammatory response in the lung. As expected, a negative association between particle diameter and inflammatory response was reported. It can be speculated that the smaller the particle size is, the deeper it can penetrate into the lungs and reach the alveolar regions, exerting more inflammatory effects than larger particles. Interestingly, it was not the particle size, rather the specific surface area that strongly correlated with the magnitude of pulmonary inflammation in the aforementioned study.

The initial stage of atherogenesis involves endothelial dysfunction and there is increasing evidence to suggest an association between impaired endothelial function and cardiovascular events. Although the detailed mechanisms are far from being understood, there is indirect evidence to support a link between inflammation and endothelial dysfunction. Intriguingly, periodontitis, a highly prevalent inflammatory disease, has been shown to be associated with endothelial dysfunction, and the treatment of this condition has been reported to improve endothelial function.

In conclusion, we investigated the effects of CB dispersion into the trachea on the development of atherosclerosis in LDLR/KO mice under a 0.51% Chol diet, and confirmed that CB exposure resulted in acceleration of development of atherosclerosis. The present study may confirm a possible link between air pollution from automobile exhaust and atherothrombotic disease. Future studies involving ambient exposures at lower concentrations for a longer period will be required to infer the human health risks of fine particulate matter in the environment.

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**References**


