Particulate matter air pollution exposure promotes recruitment of monocytes into atherosclerotic plaques

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Yatera K, Hsieh J, Hogg JC, Tranfield E, Suzuki H, Shih CH, Behzad AR, Vincent R, van Eeden SF. Particulate matter air pollution exposure promotes recruitment of monocytes into atherosclerotic plaques. Am J Physiol Heart Circ Physiol 294: H944–H953, 2008. First published December 14, 2007; doi:10.1152/ajpheart.00406.2007.—Epidemiologic studies have shown an association between exposure to ambient particulate air pollution <10 μm in diameter (PM10) and increased cardiovascular morbidity and mortality. We previously showed that PM10 exposure causes progression of atherosclerosis in coronary arteries. We postulate that the recruitment of monocytes from the circulation into atherosclerotic lesions is a key step in this PM10-induced acceleration of atherosclerosis. The study objective was to quantify the recruitment of circulating monocytes into vessel walls and the progression of atherosclerotic plaques induced by exposure to PM10. Female Watanabe heritable hyperlipidemic rabbits, which naturally develop systemic atherosclerosis, were exposed to PM10 (very late antigen-4 chain), and increased expression of CD54 (ICAM-1) and CD106 (VCAM-1) in plaques. Exposure to PM10 caused progression of atherosclerotic lesions in thoracic and abdominal aorta. It also decreased circulating monocyte counts, decreased circulating monocytes expressing high levels of CD31 (platelet endothelial cell adhesion molecule-1) and CD49d (very late antigen-4 α-chain), and increased expression of CD54 (ICAM-1) and CD106 (VCAM-1) in plaques. Exposure to PM10 increased the number of BrdU-labeled monocytes adherent to endothelium over plaques and increased the migration of BrdU-labeled monocytes into plaques and smooth muscle underneath plaques. We conclude that exposure to ambient air pollution particles promotes the recruitment of circulating monocytes into atherosclerotic plaques and speculate that this is a critically important step in the PM10-induced progression of atherosclerosis.

atherosclerosis; adhesion molecules

Epidemiologic studies have associated exposure to ambient particulate air pollution <10 μm in diameter (PM10) with increased morbidity and mortality of cardiovascular diseases (5, 8, 10, 23, 40, 54). Those with greater cardiovascular vulnerability, such as diabetic patients and the elderly, were found to be particularly susceptible to the effects of urban air pollution (5, 8, 56). However, the mechanism by which PM10 induces these adverse cardiovascular episodes remains unclear and is an area of active investigation.

Seaton et al. (41) proposed that deposition of ultrafine particles in the lung provokes alveolar inflammation that results in acute changes in blood coagulability, leading to morbidity and mortality of cardiovascular disease. Several studies from our laboratory (19, 48–50) supported this inflammatory hypothesis, showing that exposure to ambient PM10 promotes inflammation in the lung and is associated with a systemic inflammatory response. Alveolar macrophages (50) and lung epithelial cells (13, 14) incubated with PM10 release significantly higher amounts of cytokines and chemokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-6, IL-8, and macrophage chemoattractant protein (MCP)-1. Higher levels of these circulating cytokines (IL-6, IL-1β) have been observed in humans exposed to PM10 particles (48) and were associated with bone marrow stimulation characterized by an increase in circulating levels of band cells. These observations were supported by animal studies showing increased production and release of polymorphonuclear leukocytes (PMN) and monocytes (19, 49) from the marrow after PM10 exposure.

The concept that exposure to PM10 is associated with a systemic inflammatory response has been supported by other studies showing an increase in C-reactive protein (CRP) levels (36, 39), a biomarker of cardiovascular diseases. We recently demonstrated (45) that chronic exposure to PM10 causes downstream vascular effects that result in progression of atherosclerosis, and this has recently been confirmed in human studies (25). PM10 exposure also induces phenotypic changes in plaques characteristic of plaque vulnerability and instability (45).

Atherosclerosis is an inflammatory disease of vessel walls in which monocytes play a pivotal role in its pathogenesis (31, 38, 47). Monocytes migrate into the vessel wall, where they differentiate into macrophages and express scavenger receptors that allow them to phagocytose modified lipoproteins. This internalization causes the cells to develop into foam cells and causes the release of cytokines that perpetuate the inflammation and lipid accumulation, leading to the progression of atherosclerosis and augmenting the vulnerability of plaques. In the process of monocyte recruitment, changes in adhesion molecules on monocytes are involved in leukocyte tethering, rolling (E- and L-selectin), firm adhesion (CD11/CD18 integrins), and transmigration [very late antigen (VLA)-4, platelet endothelial cell adhesion molecule (PECAM)-1] by interacting with molecules on the endothelium (intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule...
(VCAM)-1]. The expression of these various surface antigens is modulated by inflammatory cytokines (22, 28, 35, 46).

We recently demonstrated (17) that exposure to urban air pollution particles (PM$_{10}$) shortens monocyte transit time through the bone marrow and rapidly releases them into the circulation in Watanabe heritable hyperlipidemic (WHHL) rabbits (53). The present study was designed to determine whether PM$_{10}$ exposure accelerates the recruitment of these monocytes into atherosclerotic plaques by transfusing whole blood (18, 20) harvested from donor animals in which dividing monocytes in the marrow have been labeled with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) into recipient animals.

**MATERIALS AND METHODS**

**PM$_{10}$ Particles**

Ambient air pollution particulates (EHC-93) were obtained from the Environmental Health Directorate, Health Canada. They were collected over Ottawa, ON, Canada in 1993 by passing 100% outside air through Videlon bag filters with a nominal cutoff of 0.3 μm from a single-pass air filtration system. The particles collected by vacuuming were then passed through a 100-μm-mesh Nytex monofilament filter. The particles had a mean diameter of 0.8 ± 0.4 μm (±SD), consisted of 99% particles by number, and contained coarse particles (2.5–10 μm in diameter) and fine particles (<2.5 μm in diameter). EHC-93 contained mainly fine particles, which composed 20% of the mass. Details about elemental composition and solubility of EHC-93 are described elsewhere (1, 20, 52). The endothoxin level in EHC-93 (0.096 endotoxin unit/ml) was too small to induce a systemic inflammatory response when instilled into the rabbit lungs (51).

**Animals**

This study was approved by the Animal Experimentation Committee of the University of British Columbia and was based on 31 female WHHL rabbits (53) with an average weight of 3.2 ± 0.1 kg (Covance Research Products, Denver, PA). All animals were 42 wk old at the start of the experimental protocol and were fed standard rabbit chow.

**Experimental Protocol**

**Exposure to PM$_{10}$.** The experimental animals ($n = 15$) were exposed by depositing the PM$_{10}$ suspended in saline in the lung by intratracheal instillation, and the control animals ($n = 16$) were treated in the same way with saline alone. The EHC-93 suspension was sonicated before instillation to ensure even distribution and prevent aggregation of the particles. The rabbits were anesthetized with 5% isoflurane, and 1 ml of normal saline or PM$_{10}$ (5 mg EHC-93 mixed with 1 ml saline) was instilled twice a week for 4 wk, as previously described (29, 45). One milliliter of blood was collected with 22-gauge needles into Vacutainers containing ethylenediaminetetraacetic acid (EDTA) from the central ear artery twice before instillation for analysis of rabbit blood cell counts. The thymidine analog BrdU (Sigma, St. Louis, MO) was injected intravenously at a dose of 100 mg/kg every 2 h for a total of five injections. This results in BrdU labeling of ~80% of the monocytes present in whole blood harvested 24 h after the first BrdU injection, and <3% of BrdU-labeled cells in the blood sample are other than monocytes (PMN and lymphocytes) (18). These labeled cells were transfused into serum-compatible WHHL recipient rabbits in whole blood as previously described (20). Three days after the transfusion the recipient rabbits were killed with an overdose of pentobarbital, and thoracic and abdominal aorta were harvested.

**Circulating Leukocyte Counts**

White blood cell (WBC) counts and differential leukocyte counts were obtained from analysis of EDTA blood with an Abbott Diagnostics Cell-Dyn 3700 instrument (Abbott Park, IL) calibrated for analysis of rabbit blood cell counts.

**Flow Cytometric Analysis**

The expression of CD11a (mouse anti-rabbit CD11a Mab NR185; Celltech R&D, Slough, UK), CD11b (mouse anti-rabbit CD11b MAb 198; Celltech R&D), CD18 (mouse anti-human CD18 MAb 60.3; Bristol-Myers Squibb, Seattle, WA), CD31 (mouse anti-human CD31 Mab IC70A; DAKO Laboratories, Copenhagen, Denmark), CD49d (mouse anti-human CD49d MAb HP2/1; Serotec), and CD62L (MAB DREG 200; kind donation of Dr. C. C. Butcher, Stanford University School of Medicine, Stanford, CA) on circulating monocytes was determined by two-color immunofluorescent flow cytometric analysis. Monocytes were recognized based on the expression of CD14 (mouse anti-human CD14 Mab Tuk4; Santa Cruz Biotechnology, Santa Cruz, CA) and typical gating of mononuclear cells. Briefly, 50 μl of whole blood in EDTA was incubated with R-phycocerythrin (RPE)-conjugated Mab Tuk4 (0.5 μg/ml) and a MAb specific for an adhesion molecule for 30 min in the dark at room temperature. The FITC-conjugated MAbs used were NR185 (1 μg/ml), 198 (5 μg/ml), 60.3 (5 μg/ml), IC70A (5 μg/ml), HP2/1 (5 μg/ml), and DREG 200 (5 μg/ml). After incubation, cells were washed with PBS-EDTA (pH 7.3), centrifuged at 1,200 rpm for 10 min (Beckman Coulter Allegra 6), and aspirated down to 300 μl. The cells were then incubated with FITC-conjugated secondary antibody for 30 min (goat anti-mouse IgG Mab, Dakocytomation, Copenhagen, Denmark). RPE-conjugated mouse IgG2α and FITC-conjugated mouse IgG1k (Dakocytomation) were used as controls. Erythrocytes in the specimen were lysed with a whole blood lysing kit containing Immumolyse and Fixative (Beckman Coulter, Fullerton, CA). The sample was then washed with PBS and centrifuged at 1,200 rpm for 10 min, and the supernatant was discarded. Cells were fixed with 1% paraformaldehyde and stored at 4°C. Mononuclear cells were recognized in the flow cytometer (Coulter EPICS XL-MCL) based on typical forward- and side-scatter patterns. Monocytes were then distinguished based on positive expression of CD14 as indicated by high PE fluorescence intensity (FL2 channel). The FITC fluorescence intensity (FL1 channel) was evaluated for a total of 3,000 cells per specimen, and results were reported as mean fluorescent intensity (MFI) after color compensation.

**Immunohistochemistry**

Samples of thoracic and abdominal aorta were selected in a systematic random fashion, frozen in liquid nitrogen, and stored at −80°C until use. Frozen tissue was mounted on a Jung Frigocut 2800N cryostat (Leica, Wesler, Germany), where 6-μm sections were cut onto slides, air dried, fixed in acetone for 10 min at −20°C, and incubated with serum-free protein block (Dakocytomation) for 15 min in a humidity chamber at room temperature. Tissue was stained for ICAM-1 (mouse anti-ICAM-1 Rhb2/3) and VCAM-1 (mouse anti-VCAM-1 Rbl/9) (generous gifts from Dr. Myron I. Cybulsky, University of Toronto). Tissue was incubated with either a 1:10 dilution
of Rb2/3 in PBS and 5% BSA or a 1:20 dilution of Rb1/9 in PBS and 5% BSA for 60 min at room temperature in a humidity chamber. Unbound antibody was removed with three washes of Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl, pH 7.6) of 5 min each. Antibody binding was visualized with the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. A 1:20 dilution of rabbit anti-mouse IgG (DAKO Laboratories) was applied for 30 min, washed in TBS and 0.1% Tween 20 followed by a 1:50 dilution of IgG alkaline phosphatase-conjugated complex (DAKO Laboratories) for 30 min, and then developed in 50 ml of TBS (pH 8.7) containing a mixture of 0.25 ml of 4% sodium nitrite, 0.1 ml of 5% fuschin (Merck, Rahway, NJ) in 2 N HCl, and 25 mg naphthol AS-B1 (Sigma) dissolved in 300 ml of N,N-dimethylformamide for 20 min. Endogenous alkaline phosphatase was blocked by the addition of 50 μg of levamisole (Sigma). The sections were then counterstained with Gilson’s modified hematoxylin. The images of all arterial sections were captured with a Spot camera, and specific antibody binding was quantified by the hue saturation intensity technique of color image analysis with ImagePro-Plus version 4.0 software on randomly selected fields of view in a blinded fashion.

**Circulating BrdU-Labeled Monocytes in Donor and Recipient Rabbits**

The number of BrdU-labeled monocytes (MoBrdU) in the peripheral blood was determined with a flow cytometric method and CD14 (Tuk4, RPE conjugated, Dakocytomation) to identify monocytes and BrdU (Bu20a, FITC conjugated) to identify labeled cells according to the method we previously developed and described in more detail (42). Briefly, whole blood in acid-citrate-dextrose was diluted 1:1 in buffer (PBS, 2 mM EDTA with 0.5% BSA), layered over Histopaque-1077 (Accuspin System, Sigma Diagnostics, St. Louis, MO), and centrifuged at 1,350 rpm for 50 min to separate mononuclear cells from the whole blood. The mononuclear cell layer was collected, washed with PBS-EDTA-BSA twice, and incubated with RPE-conjugated CD14 (Tuk4, 0.2 μg/ml) in the dark at room temperature for 15 min. The cells were washed and incubated with Microbead-conjugated anti-PE MAb (Miltenyi Biotec, Auburn, CA) in the dark at 4°C for 15 min, and magnetic separation was performed with the AutoMACS system (Miltenyi Biotec) to separate monocytes from monocellular cells. The DNA of monocytes was denatured in 2 N HCl and pepsin (0.2 mg/ml) for 30 min and neutralized with 0.1 M borate buffer (pH 8.5), and the nuclei were suspended in PBS with 2% BSA and 0.5% Tween 20 and then incubated with FITC-conjugated Bu20a (0.5 μg/ml) in the dark for 30 min at 4°C. FITC-conjugated mouse IgGk was used as a control. The nuclei were then washed, stained with propidium iodide (5 μg/ml), and stored at 4°C until analysis. The nuclei were subjected to flow cytometric analysis on the EPIC XL-MCL. Apoptotic and aggregated nuclei were gated out based on intensity of propidium iodide fluorescence and forward scatter patterns, and MoBrdU were considered to be those nuclei that stained positive for FITC compared with control.

**Calculation of Half-Life of MoBrdU in Recipients**

The exponential decrease of the number of MoBrdU in the circulation is described by the equation $N_t = N_{max} \times e^{-kt}$, where $T_{max}$ is the time at which the maximal number of MoBrdU are in the circulating blood of recipients after transfusion, $k$ is the positive rate of loss of MoBrdU (slope), $t$ is time after $T_{max}$, $e$ is 2.71828, $N_t$ is the number of MoBrdU in the circulation at time $t$, and $N_{max}$ is the number of MoBrdU in the circulation at time $T_{max}$. Because the half-life ($t_{1/2}$) can be estimated as the time at which $N_t$ is $N_{max}/2$, the late-decay equation for $t_{1/2}$ becomes $t_{1/2} = \ln 2/k$. The constant $k$ was calculated with the random-effects regression method, and the confidence interval (6) was obtained by deriving the lower and upper bounds of the 95% confidence interval for the slope, $k$.

**Quantification of Aortic Atherosclerosis**

The surface area of aorta covered by plaque was quantified macroscopically by en face two-dimensional point counting with $\times 4$ magnifying lens and also three-dimensionally quantified microscopically by standard quantitative histological methodology described in detail previously (45). Thoracic and abdominal aorta was fixed in 10% phosphate-buffered formalin for 24 h and divided into four roughly equal parts that were randomly sampled to obtain three tissue samples from each part. Samples were processed through paraffin embedding, and sections were stained by the Movat method. Random fields of view were selected and electronically captured by a Spot camera, and the volume fraction of atherosclerotic plaque was determined in a blinded fashion with point counting and ImagePro-Plus version 4.0 software.

**Quantifying BrdU-Labeled Monocytes in Aortic Tissue**

**Tissue sampling and staining for MoBrdU.** The randomly selected paraffin-embedded tissue blocks from both thoracic and abdominal aorta (see above) were sectioned at 4-μm thickness and stained for the presence of nuclear BrdU with anti-BrdU MAb Bu20a (DAKO Laboratories) and the APAAP method to identify BrdU-positive cells as described previously in more detail (4).

**Quantifying MoBrdU in vessel walls.** The cross sections of the aorta were divided into five regions: 1) endothelium over normal vessel, 2) endothelium over plaque tissue, 3) plaque tissue, 4) muscle layer under normal endothelium, and 5) muscle layer under plaque tissue (see Fig. 6). The number of MoBrdU in each compartment was determined by standard quantitative morphometric analysis (9, 21). Briefly, the number of MoBrdU in sections was determined with a modification of sequential-level stereological analysis involving a multiple-level sampling design at increasing magnifications, whereby the object phase at one level becomes the reference phase in the next level. The total aortic wall volume of each rabbit was measured as the reference for all histological studies (surface area × average thickness of aortic wall determined by ImagePro-Plus version 4.0 software). Random fields of view were captured by a Spot digital camera (Microspot, Nikon, Tokyo, Japan) and coded and examined without knowledge of the group by two observers (J. Hsieh, H. Suzuki). The volume fractions (vol/vol) of MoBrdU in the different compartments were determined by using a grid of 2,226 (42 × 53) points superimposed onto the captured image at a magnification of $\times 400$. The lengths of elastic lamina, normal endothelium, and endothelium over the plaque were measured with ImagePro-Plus version 4.0 software (Media Cybernetics, Silver Spring, MD). The density of the grid and the number of fields counted were selected to maintain the coefficient of error of the estimate of the volume below 0.1. The number of MoBrdU in each space was calculated separately to use the volume-weighted estimation of nuclear volume of MoBrdU corrected for the number of MoBrdU originally transfused and is represented as the number of MoBrdU in the volume of interest.

**BrdU Immunogold Staining for Electron Microscopy**

Tissue from the thoracic aorta was used for electron microscopy (EM) processing. Small pieces of plaque tissue were collected from the four randomly selected regions of the aorta (see above) and fixed in 4% paraformaldehyde in 0.1 M Sorensen buffer (pH 7.4) for 6 h at room temperature. Specimens were dehydrated with ethanol and immersed in LR Gold (London Resin). The resin was polymerized with 1.5% benzoyl peroxide on ice for 24 h. Ultrathin sections were cut and placed on foamvar-coated nickel grids. Tissues from four rabbits from the PM10 group and four rabbits from the control group were used, and ultrathin sections were obtained from six blocks from each rabbit. BrdU immunogold staining was similar to that for the immunohistochemical detection of BrdU described above except for three modifications. Grids bearing ultrathin sections were incubated...
with blocking solution (5% normal goat serum plus 1% BSA dissolved in 0.1 M Sörenson buffer) for 20 min. Subsequently, grids were incubated with 25 μg/ml BrdU antibody or isotype-matched IgG at respective concentrations at 4°C for 24 h before incubation with 5 μg/ml goat anti-mouse secondary antibody conjugated to 10-nm gold particles for 1 h at room temperature. Ultrathin sections were then fixed in 1% glutaraldehyde for 5 min. After immunogold staining, ultrathin sections were stained with uranyl acetate and observed with a Tecnai 12 electron microscope.

Statistical Analysis

All values are expressed as means ± SE. Data were analyzed with two-way analysis of variance (ANOVA) for repeated measures (where appropriate), and the effect of multiple comparisons was corrected with the Fisher’s protected least significant differences test as the post hoc test among the groups. If the two-way ANOVA analysis revealed a positive interaction, analysis was continued with the Student’s t-test. A one-way ANOVA was used to evaluate the difference between PM10 and control groups in terms of ICAM-1 and VCAM-1 expression.

RESULTS

Leukocytes in the Circulation

There was a decrease in circulating total WBC in the first 24 h following the first instillation of PM10 (7.16 ± 0.87 × 10⁹ vs. 9.67 ± 0.62 × 10⁹ cells/l, mean of 7 measurements over 24 h; PM10 vs. control, P < 0.05). Monocyte counts tended to be lower after the first instillation of particles; this trend became significant 24 h after the first instillation of PM10 (acute effect), and repeated exposure to PM10 (chronic effect) was not associated with a change in circulating monocyte counts (Fig. 1).

PM10-Induced Atherosclerosis

Exposure to PM10 for 4 wk resulted in an increased fraction of the aortic surface being taken up by atherosclerotic plaque (en face 2 dimensional) and an increase in the volume fraction (vol/vol) of the aortic wall taken up by plaque (3 dimensional) (Fig. 2).

Adhesion Molecule Expression on Circulating Monocytes

Figure 3 shows the short-term change in MFI ratio from the baseline of adhesion molecule expression on the surface of circulating monocytes during the 24 h following the first instillation of PM10 (acute effect). Acute exposure decreased the expression of CD31 (Fig. 3A) and CD49d (Fig. 3B) on circulating monocytes, with a trend toward a reduction of CD18 (data not shown) and no differences in CD11 or CD62L between groups. Figure 4 shows the long-term changes in MFI ratio from the baseline of adhesion molecules on monocytes induced by repeated exposure to PM10 (twice a week for 3 wk, chronic effect). Repeated exposure to PM10 decreased adhesion molecule expression on circulating monocytes, specifically CD11b (P < 0.05) and CD18 (P < 0.05). No significant difference was observed between the PM10 and control groups with regard to CD11a, CD31, CD49d, and CD62L expression (chronic effect).

Adhesion Molecule Expression in Aortic Plaques

Figure 5, A and B, show ICAM-1 and VCAM-1 expression, respectively, on atherosclerotic plaques in the aorta induced by PM10 exposure. The expression of both ICAM-1 and VCAM-1 was predominantly on the endothelial surface (confirmed with von Willebrand factor staining), but expression was also seen in plaque tissues. PM10 exposure induced more ICAM-1 expression in and on plaques than saline exposure (volume fraction 11.29 ± 2.01% vs. 4.30 ± 1.06%; PM10 vs. control, P < 0.05; Fig. 5A) and more VCAM-1 expression in and on...
plaques than in the control group (7.03 \pm 0.81\% vs. 4.36 \pm 0.94\%; PM10 vs. control, \textit{P} \leq 0.05; Fig. 5B).

\textbf{Clearance of MoBrdU from the Circulation}

The clearance of transfused MoBrdU released from the circulation was not different between the PM10- and saline-exposed recipients (data not shown), and the calculated t\(_{1/2}\) of MoBrdU was not different between groups (PM10 = 11.6 h, range 7.7–25.2 h; control = 13.8 h, range 8.3–44.1 h).

\textbf{Recruitment of MoBrdU into Vessel Walls}

Figure 6A shows the different compartments in the vessel wall where monocyte recruitment was enumerated, and representative examples of recruited MoBrdU in these compartments are shown in Fig. 6, B–D.

\textbf{Attachment of MoBrdU onto Endothelium}

In the PM10 group, significantly more MoBrdU were attached onto the endothelial surface over plaques (Fig. 7) than in the control group. There were no differences in attachment of MoBrdU onto endothelium overlying normal vessel walls (data not shown) between groups.

\textbf{Recruitment of MoBrdU into Plaques and Vessel Walls}

PM10 exposure induces significantly more recruitment of MoBrdU into atherosclerotic plaques (Fig. 8A; \textit{P} \leq 0.001) and into smooth muscle under plaque (Fig. 8B; \textit{P} < 0.01). Labeled monocytes were also seen in the muscle layer under normal endothelium, but there were no difference between groups (data not shown).

\textbf{Immunogold Staining}

Figure 6E shows a foam cell underneath the endothelium, and Fig. 6F shows immunogold staining in the nucleus of the foam cell. Nuclear staining with gold particles was found in a total of 24 cells in plaques in the PM10 group; 14 of these cells had characteristics of foam cells, 5 had macrophage morphology, and the remaining 5 had a monocytic or undetermined cell type morphologically. A total of five cells showed nuclear staining in plaques from control animals. Two of these had characteristics of foam cells, two had macrophage morphology, and the remaining cell was of undetermined origin. In BrdU-positive cells, gold particles were localized within the nucleus and no gold particles were found in cytoplasm. No staining was observed when the primary antibody was replaced with isotype-matched mouse IgG.

\textbf{DISCUSSION}

The present results confirm a previous report from our laboratory showing that exposure to ambient particulate matter (PM10) accelerates the progression of atherosclerosis over the surface of the aorta and into the aortic wall of Watanabe rabbits (45), and a recent study from another laboratory in Apo-E mice.
supports this notion (7). They extend these novel findings by showing that circulating monocytes are recruited into the plaques to a greater degree in the PM10-exposed animals than in the control animals. The recruitment of monocytes into atherosclerotic lesions is a critical step in the development and progression of atherosclerosis, and our results (Figs. 6–8) indicate that PM10 induces a greater accumulation of monocytes both in the plaques and in the smooth muscle beneath the plaques. It also shows that monocyte recruitment into plaques is associated with activation of adhesion molecules on arterial endothelium. This PM10-induced augmentation of monocyte recruitment into atherosclerotic plaques suggests that it is an important mechanism in the PM10-induced progression of atherosclerosis.

The exposure protocol used in this study results in a mean 35% of the alveolar macrophages present on the lung surface taking up particles by phagocytosis. The extent to which the alveolar macrophages take up these particles also correlates with the systemic response induced by the exposure (30, 45). We estimated that an ~2.5-kg rabbit has an alveolar surface area of 5.9 m² and that the total dose of 25.8 ng/cm² over the experimental period is similar to the levels achieved in rats exposed in chambers (51) and similar to the dose estimated for humans exposed to 150 µg/m³ for 20 days or to the exposure in a major North American city for 3 mo. This magnitude of exposure is also similar to that calculated for humans during the southeast Asia forest fires of 1997 (48). Therefore we conclude that the dose of particles delivered to the alveolar surface of these animals is comparable with other animal experiments and relevant to human exposure.

The whole blood transfusion method (18, 20) that has been developed in our laboratory does not require purification procedures and therefore avoids activation of the cells during a purification process. The protocol that we used to label the dividing monocytes in the bone marrow with the thymidine analog BrdU has been reported previously, and it labels 80% of circulating monocytes when blood is harvested from donors at 24 h from the first BrdU injection (20). By labeling the cells in donors and transfusing them into both exposed and nonexposed recipients, we are only examining the recruitment of monocytes from the blood to the aortic wall over the time period between the injection of the blood containing the labeled cells and the death of the animal (3 days). This method allows us to study the behavior of monocytes in the intravascular space and their migration into vessel walls induced by exposure to PM10. In this study we have also demonstrated that BrdU-labeled cells can be identified in tissues at the ultrastructural level (Fig. 6, E and F).

Transfusion of monocytes from PM10-exposed donors to PM10-exposed recipients resulted in the highest recruitment of monocytes on and into aortic vessel walls (Figs. 7 and 8). We have assumed that direct contact between BrdU-labeled cells and endothelium indicates that cells are adherent to endothelium (Fig. 6D) but recognize that adhesion of leukocytes to endothelium is difficult to prove in in vivo studies. The presence of a greater number of monocytes on the endothelium over the plaques (Fig. 7) and the increased number of labeled monocytes both in the atherosclerotic plaques and in the smooth muscle layer below the plaques (Fig. 8B) clearly indicate that there was an increased migration of these cells into the plaques. The increased number of cells in the plaques is consistent with the monocyte recruitment that we observed in the whole blood transfusion experiments. This increased migration suggests that PM10 induces an increased adhesion of monocytes to the endothelium over the plaques, and these cells may play a role in the progression of atherosclerosis.

Fig. 5. Volume fraction (vol/vol) of intercellular adhesion molecule-1 (ICAM-1; A) and vascular cell adhesion molecule-1 (VCAM-1; B) expression in the atherosclerotic plaques on the aorta of WHHL rabbits exposed to PM10 (n = 10) or saline (control, n = 9). The PM10-exposed group expressed significantly higher ICAM-1 and VCAM-1 in and on plaques compared with plaques from control animals. Values are means ± SE. Microphotographs (bottom) are representative examples of ICAM-1 (arrows) and VCAM-1 (arrowheads) expression patterns in plaques of rabbits exposed to PM10. Note the expression of VCAM-1 in plaque tissue.
into the atherosclerotic lesions in the animals that were exposed to PM₁₀. Furthermore, the absence of a difference in monocyte accumulation between exposed and control animals in normal vessel walls strongly suggests that plaques are particularly prone to macrophage migration induced by PM₁₀ exposure.

We also examined surface adhesion molecule expression on circulating monocytes by flow cytometry and on the aortic endothelium by immunohistochemistry, because several of these molecules have been shown to be critically important in monocyte recruitment into vessel walls (22, 28, 35, 46). The selectin group of adhesion molecules are involved in monocyte rolling and tethering to activated endothelium, while the integrins (CD18/CD11, VLA-4) are required for firm adhesion to proteoglycans and immunoglobulin molecules (ICAM-1 and VCAM-1) on the vascular endothelium. PECAM-1 (also known as CD31), which is localized at endothelial tight junctions and is expressed on monocytes, is involved in transmigration of cells into vessel walls. After acute PM₁₀ exposure (Fig. 3), our data show a transient increase (at 2 h) in the expression of CD31 and CD49d on circulating monocytes and a decrease over the next 24 h. This suggests intravascular activation of circulating monocytes following acute PM₁₀ exposure with subsequent removal of monocytes expressing high levels of CD31 and CD49d from the circulation via margination, sequestration, and recruitment. With repeated exposure to PM₁₀, expression of CD18 and CD11b (monocyte activation markers) on circulating monocytes also decreased (Fig. 4), suggesting removal of these activated monocytes from the circulation. These results are consistent with previous reports from controlled studies in humans exposed to particulate matter showing a decrease in circulation CD11b-expressing monocytes (14, 15). Circulating proinflammatory mediators induced by PM₁₀ exposure such as IL-1β and IL-6 (44, 50) may activate monocytes and decrease their deformability, thereby promoting the margination and sequestration of monocytes expressing high levels of CD18/CD11 complex.

Interestingly, in vitro studies by Becker and Soukup (3) showed that direct exposure of peripheral blood monocytes to PM₁₀ (EHC-93) causes a decrease in their CD11b and CD29 expression. Recently several studies have shown that ultrafine particulate matter (UFP) can translocate from the lung into the
peripheral blood (32–34); the biological downstream effects on circulating blood cells and blood vessels are still unclear. We speculate that translocated UFP may contribute to the decrease in circulating monocytes expressing CD11b after PM10 exposure.

Proinflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and IL-6 activate endothelium and upregulate VCAM-1, ICAM-1, and selectins (26), while TNF-α induces the secretion of MCP-1 by endothelial cells (37) to promote the migration of monocytes into atherosclerotic plaques. We showed significantly more ICAM-1 and VCAM-1 expression on endothelium cover plaques and in plaque tissue in the PM10-exposed group (Fig. 5). These molecules are known to promote monocyte adhesion to endothelium and migration into atherosclerotic plaques (2, 7, 21, 37, 44) via a mechanism that involves ligation of Mac-1 (CD11b/CD18 or M5M2) and CD49d/CD29 (VLA-4) on the monocyte surface to ICAM-1 and VCAM-1 on the endothelial cells, respectively. Proinflammatory mediators responsible for endothelial activation may originate either from the lung or from circulating leukocytes such as monocytes. Interestingly, Kristovich et al. (24) reported endothelial cell activation with increase in VCAM-1 and ICAM-1 expression by supernatant of diesel exhaust particle-exposed human peripheral blood monocytes. This suggests that circulating monocytes could also contribute to produce mediators that activate endothelial cells. Activation of endothelium is a key element in plaque instability, and expression of VCAM-1 and ICAM-1 in plaque tissue has been associated with new plaque vascularization, intraplaque bleeding, and vulnerability to rupture (2).

Potential mechanisms of how exposure to air pollution particles induces vascular disease are still largely unknown but are a area of active research and investigation. Studies from several laboratories including our own (15, 19, 32, 48–50) have demonstrated that a systemic inflammatory response is induced by exposure to ambient particulate matter exposure, supporting the hypothesis put forward by Seaton and colleagues (41). This systemic response is characterized by an increased release of PMN (49) and monocytes (19) from the bone marrow as well as elevated levels of circulating cytokines such as IL-6 and IL-1β (48), CRP (36), and procoagulation factors (15, 32). Elevated levels of these proinflammatory effector cells and mediators in the circulation have been implicated in the pathogenesis of progression and destabilization.

Fig. 7. Number of MoBrdU attached onto endothelium overlying normal endothelium or atherosclerotic plaques per millimeter of length of the endothelium of interest. Labeled PM10-exposed donor cells were transfused into PM10-exposed recipients and control donor cells transfused into control recipients. There were more MoBrdU attached to endothelium over plaques of PM10-exposed animals than control animals (*P < 0.05) but no difference in MoBrdU attachment onto normal endothelium between groups. Values are means ± SE; n = 10 (PM10) or 9 (control).

Fig. 8. Total number of MoBrdU migrated into atherosclerotic plaques (A) and smooth muscle (SM) underneath plaque and underneath normal endothelium (B) per cubic millimeter of tissue volume. Labeled PM10-exposed donor cells were transfused into PM10-exposed recipients and control donor cells transfused into control recipients. There were more MoBrdU migrated into plaques of PM10-exposed animals than control animals (*P < 0.01) and more MoBrdU migrated into SM underneath plaques of PM10-exposed animals than control animals (*P < 0.05) but no difference in MoBrdU migration into normal vessel walls between groups. Values are means ± SE; n = 10 (PM10) or 9 (control).
of atherosclerotic plaques. We have shown that alveolar macrophages exposed to EHC-93 particles produce several cytokines including TNF-α, IL-1β, IL-6, and GM-CSF in vitro (50) and that these mediators instilled into the lung stimulate the release of monocytes from the bone marrow similar to instillation of the EHC-93 itself into the lung (19). TNF-α and IL-1β are also known to activate endothelium (37, 46), and we suspect that mediators released from the lung into the circulation after PM10 exposure contribute to the activation of both circulating monocytes and vascular endothelium, thereby contributing to accelerated recruitment of monocytes into atherosclerotic plaques. Our data also suggest that the endothelium over plaques is preferentially activated, which may contribute to monocyte migration into atherosclerotic plaques induced by exposure to PM10.

The possibility that ultrafine components of PM10 might enter the circulation from the lung and activate circulating monocytes and endothelium has been proposed by recent studies (33, 34). It has been suggested that oxidative stress and inflammation induced by PM10 exposure in the lung can increase the permeability of the lung epithelium (26), promoting the translocation of UFP into the circulation. Highly reactive transition metals that are contained in PM10 (16, 51) can oxidize circulating LDL (55), and oxidized LDL has been shown to be associated with acceleration of atherosclerosis (43). We have shown that monocytes recruited into plaques became foam cells (Fig. 6, E and F), and we suspect that they contribute to the increase in lipid content of plaques induced by PM10 exposure. This is consistent with our previous finding (45) that the progression of atherosclerosis induced by PM10 is characterized by plaques containing more lipids, a feature associated with plaque vulnerability and plaque rupture (31, 38).

In summary, our results show that exposure to ambient PM10 induces progression of atherosclerotic plaques in hyperlipidemic rabbits and that this PM10-induced progression of atherosclerosis is associated with accelerated recruitment of monocytes into plaques and smooth muscle underneath the plaques. We also show that PM10 exposure preferentially activates the endothelium over the atherosclerotic lesions by upregulating the expression of the adhesion molecules involved in monocyte recruitment. These data suggest that atherosclerotic plaques are particularly vulnerable to enhanced monocyte recruitment following exposure to ambient particulate matter air pollution. Furthermore, the rapid accumulation of lipid by the cells that have migrated into the plaque indicates that they could contribute to plaque destabilization and rupture following PM10 exposure. These observations suggest a plausible mechanism for the well-established temporal association between episodes of poor air quality and the increased incidence of acute vascular events such as acute myocardial ischemia, myocardial infarction, and stroke (5, 25, 27).

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