Colonic Injuries Induced by Inhalational Exposure to Particulate-Matter Air Pollution

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Particulate matter (PM) exposure has been associated with intestinal disorders. Therefore, there is an urgent need to understand the precise molecular mechanism involved and explore potential prevention strategies. In this study, inhaled PM is shown to activate inflammatory pathways in murine colon. In a panel study, it is found that ambient PM levels are significantly associated with elevated number of fecal white blood cells in healthy subjects. Acting as a promoter, PM exposure accelerates chemical carcinogenesis-induced colonic tumor formation in a murine model. Mechanistically, RNA-seq assays suggest activation of phosphoinositide 3-kinase (PI3K)/AKT cascades in chronically PM-exposed human colon mucosal epithelial cells. Ablation of up-stream driver fibroblast growth factor receptor 4 (FGFR4) effectively inhibits inflammation and neoplasia in PM-exposed murine colons. Notably, dietary curcumin supplement is shown to protect against PM-induced colonic injuries in mice. Collectively, these findings identify that PM exposure accelerates colonic tumorigenesis in a PI3K/AKT-dependent manner and suggests potential nutrient supplement for prevention.

1. Introduction

Epidemiologic studies have shown an association between particulate matter (PM) and an increased number of adverse intestinal outcomes. The bulk of PM enters the body majorly through inhalation, and most particles are taken up by alveolar macrophages, and ultimately excreted in the feces,[1] suggesting potential contact of the intestinal tract with PM. Urban PM exposure resulted in increased intestinal permeability and gastrointestinal epithelial cell death in mice.[2] It has been observed in mice that ingestion of PM-contaminated food modified gut microbial form and function, resulting in an inflammatory response.[3] These findings establish that the GI tract might be one of the major targets of PM exposure.

Inflammation is associated with PM-induced intestinal disorders. Epidemiologic studies support the association between PM exposure and increased risk of appendicitis and hospitalization in patients with inflammatory bowel disease (IBD).[4,5] Ananthakrishnan et al. reviewed the environmental triggers of IBD, suggesting that PM or its components may alter the mucosal defense of the host and trigger immune responses.[6] In addition, activation of key inflammatory pathways in multiple organs, such as NF-κB, JNK, MAPK,[7] Nrf2[8] and STAT3,[9] have been associated with PM exposure.

Inflammation in response to environmental stress is important for wound healing, while can also promote tumorigenesis of colorectal cancer (CRC). For instance, chronic mucosal inflammation and tissue damage predispose patients to the development of CRC.[10] The four-stage colitis-driven tumor progression model, based on the intraperitoneal administration of mutagenic agent azoxymethane (AOM) and three cycles of inflammatory agent dextran sodium sulfate (DSS) administration, is a typical rodent CRC model designed to resemble chronic inflammation-associated tumor progression.[11] Since the link between PM and intestinal inflammation, and the link between chronic intestinal inflammation and CRC have been clearly demonstrated,

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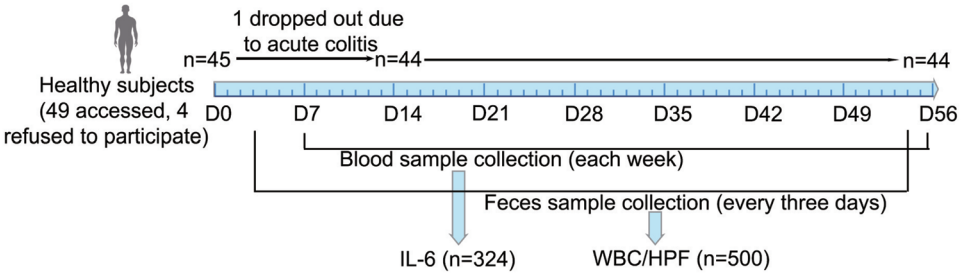

Figure 1. Scheme for the panel study to assess the association between air pollutant exposure and inflammatory response.

we hypothesized that chronic PM exposure-induced intestinal inflammation plays a key role in the increased incidence of CRC.

In this study, we evaluated pathological alterations in murine colons following chronic PM exposure, as well as potential rescue by curcumin. Subsequently, modulations in RNA expression profiles in chronic PM-exposed human colon mucosal epithelial cells were investigated with the RNA-seq assay. Our findings revealed that the potential molecular pathway/s involved in the adverse colonic outcomes in response to chronic PM exposure.

2. Results

2.1. Ambient PM Were Associated with Increased Number of Fecal WBC

A panel study was conducted to explore the association between air pollutants exposure and inflammatory response. A total of 49 participants (26 males and 23 females) were recruited, and 44 (24 males and 20 females) completed the study (**Figure 1**). The demographic characteristics of the participants including age, body mass index (BMI), plasma IL-6 levels, and the number of white blood cells (WBC)/high power field (HPF) in feces are summarized in Table S1 (Supporting Information). Table S2 (Supporting Information) summarizes the statistics on six major air pollutants throughout the follow-up period. The average concentrations of $PM_{2.5}$ and PM_{10} during the panel study period were 84.1 and 126.7 μg m^{-3}, respectively, which greatly exceeded the World Health Organization (WHO) standards (10 and 20 μg m^{-3}, respectively) or China Class II standard (35 and 70 μg m^{-3}, respectively). Simple correlation analyses revealed the $PM_{2.5}$ levels were correlated with the levels of PM_{10}, SO_2, and CO ($P < 0.0001$), but not O_3 (Table S3, Supporting Information). The levels of PM_{10} were positively correlated with the levels of NO_2, SO_2, and CO ($P < 0.0001$) (Table S3, Supporting Information).

Associations of plasma IL-6 levels or numbers of WBC/HPF in feces with concentrations of air pollutants were analyzed. Results of the single-pollutant model revealed that the levels of $PM_{2.5}$, PM_{10}, SO_2, and CO were positively associated with elevated numbers of WBC/HPF in feces. Levels of O_3 were negatively associated with elevated numbers of WBC/HPF in feces (Table S4, Supporting Information). No association between concentrations of air pollutants and plasma IL-6 levels was identified (data not shown). The levels of $PM_{2.5}$ and PM_{10} were consistently associated with increased numbers of WBC/HPF in feces with the moving average concentrations from lag 0–4 to lag 0–8 d. The highest estimated effects of both $PM_{2.5}$ and PM_{10} exposure on WBC/HPF in feces were noted following a 7 d exposure in which an IQR increase of $PM_{2.5}$ (83 μg m^{-3}) or PM_{10} (97 μg m^{-3}) was associated with increases of 1.17 (95% CI: 0.23 to 2.81) or 1.48 (95% CI: 0.33 to 3.62) WBC/HPF in feces (Table S4, Supporting Information).

In the subsequent sensitivity analysis, after adjusting for SO_2, NO_2, CO, and O_3, the estimated effects of lag 0–7 d moving average concentration of $PM_{2.5}$ were robustly associated with elevated WBC/HPF in feces (**Table 1**). Analogous estimated effects of PM_{10} have been identified in the sensitivity analysis, and after controlling for NO_2, there was no significant association between PM_{10} concentration and the number of WBC/HPF (Table 1). Taken together, the results noted above emphasize the effects of consecutive PM exposure on elevated number of WBC/HPF in feces.

2.2. Chronic PM Exposure Induced Inflammation in Murine Colons

Given the newly discovered role of PM exposure in regulating intestinal disorders, mice were continuously exposed to PM for 12 months, and pathologic alterations in colonic tissues were examined at the end of each month. PM exposure

Table 1. Cumulative increases in WBC/HPF measures associated with an IQR increase in PM concentration over lags of 0–7 d.

$PM_{2.5}$[a)]			PM_{10}[a)]		
Pollutant	WBC/HPF (95% CI)	*P* value	Pollutant	WBC/HPF (95% CI)	*P*-value
	1.12 (0.23 to 2.81)	**0.0077**		1.48 (0.33 to 3.62)	**0.0046**
+SO_2	0.39 (0.05 to 0.83)	**0.0212**	+SO_2	0.34 (0.02 to 0.77)	**0.0369**
+NO_2	0.09 (0.03 to 0.15)	**0.0022**	+NO_2	0.01 (–0.28 to 0.41)	0.9696
+CO	0.01 (0.00 to 0.02)	**0.0121**	+CO	0.01 (0.00 to 0.01)	**0.0235**
+O_3	0.82 (0.26 to 1.62)	**0.0220**	+O_3	0.39 (0.10 to 0.77)	**0.0067**

[a)]after controlling for gaseous pollutants when using two-pollutant models.

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were approved by the Committee on Animal Use and Care of Southeast University, China. All the methods in this study were performed according to approved guidelines. Mice were bred in the specific pathogen-free class laboratory animal housing of Southeast University. Five mice were housed in each polycarbonate cage with ad libitum access to food and water. Light cycles were set on a 12/12 h light/dark cycle, and room temperature was set at 22.5 °C.

Animal Treatment: Dynamic inhalation exposure chambers were outfitted with extensive air quality monitoring equipment and an aerosol generator (Beijing HuiRongHe Technology Co., Ltd, China). The concentration of suspended PM was measured gravimetrically by a real-time dust monitor (CEL-712 Microdust Pro, CASELLA CEL Inc., USA. Exposure was carried out in stainless steel Hinners-type whole-body inhalation chambers; the treatment groups received PM, and the control received high efficiency particulate air (HEPA)-filtered room air (FRA) at the same flow rate. The mice were exposed in their respective chambers for 2 h per day (10–12 a.m.), 5 d (from Monday to Friday) per week, with a mean mass concentration of PM 0.4 mg m^{-3}.

The first batch of animal experiments included two groups (with 120 C57BL/6 mice in each group): control mice exposed to FRA or mice exposed to PM. Mice were exposed to PM for 12 consecutive months. At the end of each month, blood samples of 10 mice from each group were collected from the angular vein, and then mice were sacrificed. Images of the colorectal tissues were taken, and the lengths of the colorectal tissues were recorded. Next, 4 of 10 colorectal tissues were stored in liquid nitrogen, while the other six colorectal tissues were fixed "Swiss-roll" style in 4% paraformaldehyde (PFA) at 4 °C.

A second batch of mice were divided into five groups (with 50 C57BL/6 mice in each group): control mice treated with five repeated intraperitoneal injections of vehicle (PBS) (one injection per week) and exposed to FRA (vehicle/FRA); mice treated with five repeated intraperitoneal injections of AOM (7.5 mg kg^{-1} body weight, single injection per week) and exposed to FRA (AOM/FRA); mice treated with a single injection of vehicle (PBS) and exposed to PM (vehicle/PM); mice treated with a single injection of AOM and exposed to PM (AOM/PM); the positive control mice treated with a single injection of AOM (7.5 mg kg^{-1} body weight) and then DSS (1.5% (wt/vol) in drinking water) was administered for 5 d followed by 16 d of a water regimen for three separate cycles (AOM/DSS).[11]

A third batch of mice included four groups (with 40 mice in each group): wild type (WT) mice exposed to FRA or PM; $Fgfr4^{-/-}$ mice exposed to FRA or PM.

A fourth batch of mice included four groups (with 50 mice in each group): wild type (WT) mice or $Fgfr4^{-/-}$ mice treated with a single injection of vehicle (PBS) and exposed to FRA; wild type (WT) mice or $Fgfr4^{-/-}$ mice treated with a single injection of AOM and exposed to PM.

A fifth batch of mice included four groups (with 40 C57BL/6 mice in each group): mice exposed to FRA with common or curcumin-enriched (0.2% wt/wt) diets;[35] mice exposed to PM with common or curcumin-enriched (0.2% wt/wt) diets.

For the second and fourth batches of animal experiments, mice were exposed to FRA or PM for 12 consecutive months. Half of the mice from each group (20 mice) were randomly selected for observation of their overall survival for up to 12 months. Kaplan-Meier (KM) survival plots were drawn according to the date of death of mice. For the other 30 mice from each group, the checkpoints were set from 3 to 12 months. At the end of each month, two mice were randomly selected and sacrificed for the macroscopic and histological examination of colorectal tissues. Images were taken of all colorectal tissues, and the length of the colorectum, as well as the number of tumors, were recorded. Colorectal tissues were stored in liquid nitrogen or fixed "Swiss-roll" style in 4% PFA at 4 °C.

For the third and fifth batches of animal experiments, mice were exposed to FRA or PM for 12 consecutive months. At the end of 3, 6, 9, and 12 months, 10 mice were randomly selected and sacrificed for the macroscopic and histological examination of colorectal tissues. Colorectal tissues were stored in liquid nitrogen or fixed "Swiss-roll" style in 4% PFA at 4 °C.

Histopathological Analysis of Mice Lung Tissues: PFA fixed tissues were embedded in paraffin serially sectioned (5 µm) and stained with hematoxylin and eosin (H&E). Sections were stained with Alcian blue (pH 2.5) to demonstrate acidic (sulfated and carboxylated) mucins or periodic acid Schiff (PAS) to characterize the neutral mucins, which were used to evaluate the mucodepletion in murine colon.

The epithelial injury score and inflammatory infiltration score were defined as follow: the epithelial injury score is the sum of the scores of gland mucodepletion plus scores of tissue damage, which are scored on a scale of 0 to 3 (mucodepletion: 0, none; 1, mild; 2, moderate; 3, severe; tissue damage: 0, no mucosal damage; 1, discrete epithelial lesions; 2, surface mucosal erosion or focal ulceration; 3, extensive mucosal damage and extension into deeper structures of the bowel wall).

The inflammatory infiltration score is the sum of infiltration score plus the percent area of each section. Infiltration was scored on a scale of 0 to 3 (0, no inflammatory cells; 1, infiltration around crypt bases; 2, infiltration of muscularis mucosa; 3, infiltration of submucosa), and the percent area of each section was scored on a scale of 0 to 4 (0, no involvement; 1, ≤ 25%; 2, ≤ 50%; 3, ≤ 75%; 4, ≤ 100%).

Scores were determined for the proximal, middle, and distal colon. Total scores are the sum of the scores of each segment. Histological scoring of tissues was performed by two experienced pathologists in a blinded manner.

Immunohistochemistry Staining: After dewaxing, immunohistochemistry staining were performed as described,[36] and incubated overnight at 4 °C with mouse monoclonal antibodies against FGFR4 (1:50) (Abcam, USA), p-Akt (1:100) (Cell Signaling Technology, USA), p65 (1:500) (Abcam, USA), p-STAT3 (1:100) (Abcam, USA), Ki67 (1:100) Abcam, USA). Antibody binding to tissue sections was visualized with a biotinylated rabbit anti-mouse IgG antibody (1:400; DAKO) and developed with diaminobenzidine (DAB) as a substrate. For the negative controls, the primary antibodies were omitted. Each section was examined under microscopy by two histologists using a semiquantitative immunoreactivity score (IRS) methods as follow: Category A documented the intensity of immunostaining as 0–3 (0, negative; 1, weak; 2, moderate; 3, strong); category B documented the percentage of immunoreactive area as 1 (0%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). IRS resulted from the multiplication of category A and B of each high-power field (HPF) (× 400 magnification). Each tissue condition was counted in three non-overlapping HPFs.

ELISA: The levels of inflammatory cytokines in plasma were measured using a Human IL-6 ELISA Kit (Multi Sciences, China). The levels of IL-6, TNF-α, and IL-1β in murine plasma were measured by a Mouse IL-6 ELISA kit, Mouse TNF-alpha ELISA Kit and Mouse IL-1β ELISA Kit (Multi Sciences, China) according to the manufacturer's instructions. Each sample was assayed in duplicate.

qRT-PCR Assay: The mRNA expression levels were determined by reverse transcription of total RNA followed by qRT-PCR analysis on a Quant Studio 6 Flex system (Applied Biosystems, Life Technologies, USA) using SYBR PCR Master Mix reagent kits (Toyobo). Primer sequences are shown in Table S6 (Supporting Information), and all experiments were performed in duplicate. The mRNA levels provided here are relative to CycA for the indicated gene.

Expression Plasmid Construction and Stable Lentivirus Transduction: Firefly luciferase cDNA harboring lentiviral (LTV) expression shuttle vectors was constructed and stably transduced into NCM460 cells as described.[37]

FGFR4 shRNA lentiviruses were generated by cotransfection with packaging plasmids, pSPAX2 and pMD2G. The shRNA lentivirus harbored a short-hairpin RNA sequence to target genes. NCM460 cells were added with lentivirus (MOI = 30) and treated with Blasticidin S for 2 weeks to obtain the stable transduction into NCM460 cells.

Chronic Treatment of NCM460 Cells with PM: Wild type (WT) or FGFR4 KO NCM460 cells (1×10^6) were seeded into 10 cm (diameter) dishes for 24 h. Cells were maintained in 0 (control) or 100 µg mL^{-1} PM for 48–72 h per passage. This process was continued for 30 passages.

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