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Anti-inflammatory potential of alginic acid from *Sargassum horneri* against urban aerosol-induced inflammatory responses in keratinocytes and macrophages

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ABSTRACT

The airborne particulate pollutants originating in the deserts of Mongolia and China which becomes contaminated with industrial effluents and traffic emissions while moving with the wind currents towards East Asia has recently become a serious environmental and health issue in the region. They cause asthma, collateral lung tissue damage, oxidative stress, allergic reactions, and inflammation. The current study was undertaken to evaluate the protective effects of alginate extracted from the invasive alga *Sargassum horneri* (SHA) against fine dust collected from Beijing, China (Chinese fine dust; CFD). It was found that CFD induces inflammation in HaCaT keratinocytes and inhibits macrophage activation. All of the particulate matter (PM) comprising CFD was < PM13 majority being < PM2.5 which is defined for mineral elements and polycyclic aromatic hydrocarbons. SHA attenuated PGE_2 levels in CFD-induced HaCaT keratinocytes. The IC_{50} for SHA was 36.63 ± 4.11 μg mL^{-1}. SHA also reduced the levels of COX-2, IL-6, and TNF-α, and inhibited certain key molecular mediators of the NF-κB and MAPK pathways in keratinocytes. SHA substantially reduced the levels of CFD-derived metal ions like Pb^{2+} and Ca^{2+} in keratinocytes attributable to its metal ion chelating properties. CFD-induced HaCaT keratinocyte culture media increased inflammatory responses in RAW 264.7 macrophages. These cells presented with increased levels of NO, iNOS, COX-2, PGE_2, and pro-inflammatory cytokines. It was found that the aforementioned effects could be reversed in RAW 264.7 macrophages when keratinocytes were treated with SHA. Therefore, SHA could be used against fine dust-induced inflammation in keratinocytes.

1. Introduction

Fine dust (FD) events have become a serious environmental issue in China, Korea, and Japan. According to a recent study, dermal exposure to the polycyclic aromatic hydrocarbons (PAHs) in FD could induce carcinogenesis (Strandberg et al., 2018). As reviewed by Kim et al. (2016), the toxic substances in fine dust cause conjunctivitis and inflammatory skin conditions and stimulate the release of pro-inflammatory cytokines which trigger allergic reactions. In springtime, the Korean peninsula is covered by dust clouds originating in the deserts of Mongolia and China. This natural phenomenon is known as “Asian dust”. It arrives in Korea and Japan after having passed through heavily industrialized areas of eastern China (Kwon et al., 2002). In this way, the airborne particles become contaminated with industrial pollutants. According to Raloff (2001), FD contains sulfur, soot, carbon monoxide, heavy metals (mercury, cadmium, chromium, arsenic, lead, zinc, and copper) and carcinogens such as asbestos, silica, hydrocarbons, herbicides, combustion products, and plastic ingredients such as phthalates. Currently, there is increased public awareness concerning FD. Extensive studies have been conducted on their effects on biological systems (Pozzi et al., 2003; Riva et al., 2011; Schaumann et al., 2004; Tao et al., 2003). Our previous study indicated that FD (ERM-CZ100 and ERM-CZ120) collected from a road tunnel in Wislostrada, Poland induced inflammatory responses in HaCaT keratinocytes including the production of cyclooxygenase (COX) − 2, prostaglandin E2 (PGE_2), IL-1β, and IL-6. The addition of keratinocyte culture to RAW 264.7 macrophages induced inflammatory responses in them including the production of inducible nitric oxide synthase (iNOS), nitric oxide (NO), PGE_2, and pro-inflammatory cytokines. These inflammatory responses were effectively reduced by the addition of diphlorethohydroxycarmalol, a phlorotannin isolated from *Ishige okamurae* (Fernando et al., 2017a).

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Marine brown algae contain numerous bioactive polysaccharides. Alginate is a linear copolymer composed of (1 − 4)-linked α-*L*-guluronate (G) and its C-5 epimer, β-*D*-mannuronate (M). Alginate forms hydrogels, chelates metals, and may have antioxidant and anti-inflammatory properties (Fernando et al., 2017c; Sarithakumari et al., 2013). Alginic acid purified from *Sargassum wightii* demonstrated anti-inflammatory and antioxidant potential in adjuvant-induced arthritic rats by reducing paw edema and the levels of cyclooxygenase, lipoxygenase, and myeloperoxidase. Moreover, it was reported to reduce lipid peroxidation and enhance antioxidant enzyme activity (Sarithakumari et al., 2013). In the present study, we evaluated the ability of urban aerosols (fine dust) for their ability to induce inflammatory responses in keratinocytes and, by extension, in macrophages. The fine dust was collected from Beijing, China over a 10-y period (1996–2005; Certified Reference Material No. 28). Alginate was extracted from *Sargassum horneri* collected off Jeju Island, South Korea, and evaluated for its ability to mitigate inflammatory responses elicited by fine dust.

2. Materials and methods

2.1. Materials

Urban aerosols (CRM No. 28) were obtained from the National Institute for Environmental Studies, Ibaraki, Japan. The alginate reference standard, 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH2-DA), and 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. RAW 264.7 macrophages and HaCaT cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Cytokine kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA), R&D Systems (Minneapolis, MN, USA), Becton Dickinson & Co. (Franklin Lakes, NJ, USA), and Invitrogen (Carlsbad, CA, USA). All other chemicals and solvents used were obtained from commercial sources and were the highest purity grade.

2.2. Sample collection and alginic acid purification

S. horneri samples were collected from the Seongsan coastal area of Jeju Island, South Korea. They were washed to remove salts and debris, lyophilized, and ground into powder. Alginic acid was extracted according to the method of Fernando et al. (2017b), with some minor modifications. The dry algae powder was depigmented first with hexane then with 95% ethanol. The depigmented powder was immersed in 10% formaldehyde for 8 h, filtered through a wire sieve, and washed with 95% ethanol to remove any remaining formaldehyde. The powder was then air-dried and lyophilized. It was then immersed in dilute HCl (pH 4.0). HCl was added as needed to restore the pH of the suspension to 4.0. The suspension was agitated at 30 °C for 24 h, filtered through a wire sieve, and washed with cold distilled water (DW). It was then immersed in 5% Na_2CO_3 (w/v) and agitated at 30 °C for 24 h to extract the alginic acid. The extract was filtered through a wire sieve and centrifuged at 10,000 × *g* at 4 °C for10 min. The supernatant was then recovered. The mixture was adjusted to pH 6.0 by gradual additions of HCl. A saturated $CaCl_2$ solution was added to the mixture to precipitate the alginic acid as calcium alginate. The pellet was separated by centrifugation, suspended in 10% HCl for 2 h, and recovered by centrifugation. This acid wash was repeated 6 × . After the final washing, the pellet was suspended in DW and neutralized with NaOH. The alginate solution was extensively dialyzed to remove excess ions and lyophilized to obtain *S. horneri* alginate powder (SHA).

2.3. Spectroscopic analysis (FTIR, ICP-OES, and GC-MS/MS)

Fourier-transform infrared spectroscopic (FTIR) analysis of the alginate was performed with a Thermo Scientific NicoletTM 6700 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA USA). Potassium bromide (KBr) pellets were cast by combining 5 mg sample with 5 g KBr powder. The metal ions in the fine dust and the cells were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) with a PerkinElmer OPTIMA 7300DV inductively coupled plasma spectrometer system (PerkinElmer, Inc., Waltham, MA, USA). It was calibrated with a multi-element standard (PerkinElmerN9300233) containing 10 μg mL^{-1} (10 ppm) each Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, and Zn. Each element was detected by using ≥ 2 non-overlapping wavelengths (Fernando et al., 2017c). The harvested cells were dried, dissolved in concentrated HNO_3, and mixed with H_2O_2. The acid digests were diluted in ultrapure deionized water prepared by a Milli-Q® Integral Water Purification System (MilliporeSigma, Burlington, MA, USA). Polycyclic aromatic hydrocarbon (PAH) contaminants in the fine dust and cells were analyzed by a Shimadzu GCMS-TQ8040 system (Shimadzu Corp., Kyoto, Japan). It was fitted with the Rtx-5MS fused-silica capillary column. The operating conditions were: sample injection in splitless mode; injector port temperature: 300 °C; GC oven program: hold at 90.0 °C for 2 min, then increase to 320.0 °C at 5.0 °C min^{-1}, then hold for 12 min. The carrier gas was helium at a constant flow rate of 43.7 cm s^{-1}. The harvested cells were washed in phosphate-buffered saline (PBS) and suspended in chloroform. The chloroform layer was allowed to settle, dried with anhydrous sodium sulfate, and used in the analysis.

2.4. Cell culture

RAW 264.7 mouse macrophages and HaCaT (human keratinocytes) were maintained in DMEM with 10% FBS and 1% antibiotics. The cultures were kept in a humidified atmosphere at 37 °C with 5% CO_2. The cells were subcultured periodically, and those with exponential growth were used in the subsequent experiments (Fernando et al., 2017a).

2.5. Evaluation of inflammatory responses

This experimental procedure is depicted in Fig. 1. First, HaCaT keratinocytes were seeded in 24-well plates and incubated for 24 h. Various concentrations of SHA were then added to different wells. After 30 min, Chinese fine dust (CFD) was added to the cells at 125 μg mL^{-1} (this optimum CFD concentration and exposure time was selected based on preliminary studies on cell viability and inflammatory marker production). The cells were then incubated for 30 min. The wells were rinsed twice then replenished with fresh DMEM. The culture plates were incubated at 37 °C for another 24 h in a humidified atmosphere supplemented with 5% CO_2. The cell culture was harvested to analyze the inflammatory mediators and the pro-inflammatory cytokines, and to treat the RAW cells. Cell viability was measured with a MTT assay and intracellular ROS levels were evaluated by a DCF-DA assay (Wang et al., 2017). The cells were also harvested to measure the PAHs and metal ions. The HaCaT keratinocyte medium was transferred to pre-seeded RAW macrophage culture plates and incubated at 37 °C for 30 min. The wells were then rinsed and replenished with fresh DMEM. After 24 h of incubation, the culture medium was recovered to analyze the inflammatory mediators and the pro-inflammatory cytokines.

2.6. Western blot analysis

Western blot analysis was carried out to determine the expression levels of several key molecular mediators. CFD-induced cells were harvested after a 30-min incubation to evaluate the upstream mitogen-