INTRODUCTION

As the problem of air pollution has increased in Korea, interest in the health problems caused by particulate matter (PM) is growing. PM is the sum of all the solid and liquid particles suspended in air, and this complex mixture includes both organic and inorganic particles, such as dust, pollen, soot, smoke and liquid droplets. The toxicity of PM is caused not only by its cell-permeable size but also by the combination of various substances.[1,2] Numerous epidemiological investigations into ambient air pollution have indicated that PM is correlated not only with an exacerbation of cardiovascular diseases and with an impact on respiratory system inflammation but also with the progression of inflammatory skin diseases such as atopic dermatitis, acne, psoriasis and allergic reactions.[3] In particular, high levels of air pollutants, including polycyclic aromatic hydrocarbons, heavy metals and oxides contribute to premature skin ageing.[4] A study examining 400 70- to 80-year-old German women, who resided in urban or country environments for 24 years, showed that air pollution increased skin pigmentation by 22%, and there were more wrinkles found on the skin.[5] Therefore,
it is necessary to explore the molecular mechanisms of PM-induced skin ageing.

The epidermis is the outermost protective barrier of the skin and protects the body from the external environment. The dermis is largely composed of a dense collagen-rich extracellular matrix (ECM) which supports the structure of the skin by maintaining its stiffness and elasticity. Recent studies have indicated that communication between the dermal and epidermal layers is essential for the correct function and regulation of skin homeostasis. Complex signalling networks established between the two major cellular components of the skin (keratinocytes and fibroblasts) have proven to be critical in numerous processes such as those involving skin cell growth and differentiation, tissue repair, and wound healing and in processes modulating aesthetic features such as the presence of wrinkles and firmness. Keratinocytes have been reported to synthesize and secrete cytokines such as interleukin (IL)1, IL6, IL8, tumor necrosis factor (TNF)-alpha, and transforming growth factor-alpha and -beta. There is evidence that keratinocytes stimulate fibroblasts to synthesize growth factors, which in turn will stimulate keratinocyte proliferation in a double paracrine manner. In particular, keratinocyte-derived IL1 regulates collagen and collagenase synthesis in fibroblasts depending upon the conditions.

Extrinsic skin ageing is clinically characterized by wrinkling, laxity and the acquisition of a leather-like appearance. Degradation of ECM proteins, such as collagen, fibronectin, elastin and proteoglycans, contributes to extrinsic ageing. Matrix metalloproteinases (MMPs), which are zinc-containing endopeptidases with an extensive range of substrate specificities, mediate degradation of the ECM. Recent reports have shown that decreased levels of prostaglandin E2 (PGE2), synthesized from arachidonic acid (AA) through the enzymatic activities of two cyclooxygenases (COX-1 and/or COX2), reduce MMP1 activity and consequently increases collagen density in human varicose veins.

Numerous in vitro studies have suggested that PM-induced inflammatory responses are mediated by the aryl hydrocarbon receptor (AhR) pathway and the generation of intracellular reactive oxygen species (ROS).

The aim of the present study was to investigate the mechanisms by which PM affects epidermal inflammation and skin ageing using an in vitro co-culture model of human keratinocytes and fibroblasts.

2 MATERIALS AND METHODS

2.1 Chemicals and preparation

The standard reference materials (SRMs) 1648a (pmA) and 1649b (pmB) were purchased from the National Institute of Standards and Technology and dispersed in distilled water. The pharmacologic inhibitor of p38 (SB203580) was purchased from Calbiochem. Specific siRNAs targeting AhR was purchased from Invitrogen. Specific antibodies used for Western blot analysis, including anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-c-Fos and anti-phospho-c-Jun were purchased from Cell Signaling Technology. Anti-GAPDH was purchased from Santa Cruz Biotechnology. Human IL1-alpha, IL1-beta, IL6, IL8 and TNF-alpha ELISA kits were purchased from R&D systems. Other chemicals and reagents used in this study were of analytical grade.

2.2 Cell culture and viability assay

The HaCaT cells (American Tissue Cell Collection (ATCC) obtained in 2002, USA) and HDF (Gibco BRL, Life Technologies) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. When the cultures reached confluence, the cells were treated with 0.05% trypsin/0.53 mmol/L EDTA for 5 minutes at 37°C. The cells used in these experiments were between passages 20 and 24 for HaCaT cells, and between passages 7 and 9 for HDF cells. Before experimentation, the cells were conditioned for at least 12 hours in serum-free medium.

Cell viability was determined using a water-soluble tetrazolium salt (WST-1) assay kit (EZ-CYTOX, Dogen), according to the manufacturer’s instructions. Briefly, the cells were seeded at a density of 5 × 10³ cells/well in a 96-well plate. After 24 hours, the cells were treated with different concentrations of PM (0, 10, 25, 50 and 100 μg/cm²) for 24 hours. Following this, the cells were treated with WST-1 reagent solution and incubated at 37°C for 1 hour. The absorbance values were measured at 450 nm using a microplate spectrophotometer, SpectraMax340pc (Molecular Devices). Relative cell viability was calculated by the absorbance values and normalizing the data against the untreated control group.

2.3 Western blot analysis

Standard procedures were used for Western blotting. Briefly, the cells were lysed with 1% Triton-X radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail. The remaining cell debris was removed by centrifugation (16,100 g, 15 min), and the protein concentration was determined using the bicinchoninic acid assay (BCA) method. Equal amounts of protein were loaded onto 8%-15% sodium dodecyl sulphate-polyacrylamide gels and separated by electrophoresis. After electrophoresis, the proteins were transferred onto nitrocellulose (NC) membranes. The membranes were blocked with 5% skimmed milk for 1 hour. Subsequently, the membranes were incubated with primary antibodies (1:1000 dilution) overnight at 4°C and HRP-conjugated secondary antibodies (1:2000 dilution) for 1 hour at room temperature. Proteins were detected using the EzWestLumi plus system (ATTO) and images obtained using a ChemiDoc™ XRS image analyser (Bio-Rad). Protein expression levels were quantified using Image J software and normalized to β-actin or GAPDH levels.

2.4 Intracellular ROS assay

Intracellular reactive oxygen species (ROS) levels were measured by detecting the fluorescence intensity of the oxidant-sensitive 2′,
7′-dichlorofluorescin diacetate (DCFH-DA). Briefly, DCFH-DA diffuses into cells and is de-acetylated by cellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to the highly fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF) by ROS present in the cell. Cells were seeded at a density of 5×10^3 cells/well in 96-well black plates. The medium was removed, and the cells were incubated with 20 μmol/L DCFH-DA for 30 minutes in the dark at 37°C. Subsequently, the cells were washed three times with phosphate-buffered saline (PBS) and incubated with PM in serum-free DMEM. The fluorescence intensity was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate spectrophotometer, SpectraMax340pc (Molecular Devices).

2.5 | RNA extraction and quantitative reverse transcription (RT)-PCR analysis

Total RNA was extracted from cells using the TRIzol® reagent (Welgene), according to the manufacturer’s protocol. The cDNA templates synthesized from mRNA were reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and incubated for 1 hour at 42°C. Real-time quantitative PCR assays were performed using a QuantStudio 3 system (Applied Biosystems) using a PowerUp SYBR Green Master Mix (Applied Biosystems). All data were normalized to the housekeeping gene GAPDH. Relative quantitation was analysed using the 2^−ΔΔCt method according to the manufacturer’s instructions.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Pro-inflammatory cytokines and MMP1 (both total- and pro-) levels were analysed by an enzyme-linked immunosorbent assay (ELISA). Cells were treated with PM for 24 hours. Subsequently, conditioned media was collected from the cells and stored at −80°C. Cytokines and MMP1 release by the cells were quantified using ELISA kits specific for human IL1-α, IL1-β, IL6, IL8, TNF-α, pro-MMP1 and total-MMP1 (R&D systems) according to the manufacturer’s recommended protocols.

2.7 | Statistical analysis

All data are presented as the mean ± standard error (SE). The mean values were calculated based on data from at least three independent experiment experiments that were conducted on separate days using freshly prepared reagents. Data were analysed using one-way analysis of variance (ANOVA) with multiple correction test algorithm of Bonferroni. The significance of differences was defined at a P value <0.05, and the statistical analysis was performed using SPSS (PASW statistics 18 software, IBM Co.).

3 | RESULTS

3.1 | Particulate matter induces inflammatory responses in HaCaT cells

HaCaT cells were treated with different concentrations (10, 25, 50, and 100 μg/cm²) of each PM for 24 hours. As shown in Figure 1A, significant PM cytotoxicity was observed at concentrations of 100 μg/cm² (pmA) and above 50 μg/cm² (pmB). Therefore, we selected PM
treatment concentrations of 50 μg/cm² (pmA) and 25 μg/cm² (pmB). To assess the effect of PM on inflammatory responses, AhR protein expression levels, mitogen-activated protein kinase (MAPK) and the generation of intracellular ROS were analysed. Activation of AhR was observed by examining its levels in different cell fractions following treatment with the two different PMs (Figure 1B). As a result, cytosolic AhR translocated to the nucleus following treatment with each PM. Moreover, the mRNA expression levels of CYP1A1 and CYP1B1 increased in a dose-dependent manner (Figure 1C). To analyse the generation of intracellular ROS following PM treatment, HaCaT cells were treated with different concentrations of PMs (25, 50 and 100 μg/cm²), with 45 μmol/L H₂O₂ being used as a positive control. Intracellular ROS levels were measured by detecting the fluorescence intensity of the oxidant-sensitive probe DCFH-DA. As shown in Figure 1D, PM dose-dependently induced intracellular ROS levels compared with the negative control (untreated cells). The levels of phosphorylation of p38 kinase were also increased by PM treatment (Figure 1E).

3.2 | PM induces the expression of MMP1 and COX2 in HDF cells in a co-culture model with keratinocytes

A co-culture model was designed to evaluate the factors involved in collagen degradation. To follow paracrine signalling in the absence of cell-cell contact, a permeable membrane (pore size: 0.4 μm) was inserted in the co-culture model allowing for the free diffusion of secreted soluble factors (Figure 2A). Changes in the mRNA expression levels in HDF cells, mediated by soluble factors secreted by HaCaT cells, were assessed using a Q-PCR method. The relative mRNA levels of MMP1 and COX2 were statistically increased by PM treatment in the HDF co-culture with keratinocyte (Figure 2B). To identify factors secreted by the HaCaT cells, the mRNA and protein expression levels of IL1-alpha, IL1-beta, IL6, IL8 and TNF-alpha were analysed by Q-PCR and ELISA, respectively. The cells were treated with different concentrations (0, 5, 10, 25 and 50 μg/cm²) of PM for 2 hours (mRNA) and 24 hours (protein). As shown in Figure 2C, the relative mRNA levels of IL1-alpha, IL1-beta, IL6 and IL8 were all significantly induced by PM treatment. As shown in Figure 2D, the secretion of cytokines by the HaCaT after PM treatments was identified. From these data, IL1-alpha, IL1-beta, IL6 and IL8 were selected as candidate inducible paracrine cytokines. These candidates were purchased as recombinant cytokines, and the HDF cells were treated with the concentrations of each previously determined by the ELISA. As shown in Figure 2E, the mRNA expression levels of MMP1 and COX2 in HDF were markedly induced after treatment with either IL1-alpha and/or IL1-beta, whereas IL6 and IL8 had no effect.

3.3 | The role of intracellular ROS generation and AhR activation on IL1-alpha and IL1-beta induction in PM-treated HaCaT cells

To identify the role of intracellular ROS generation and AhR activation on IL1-alpha and IL1-beta induction in PM-treated HaCaT cells, the cells were pretreated with N-acetyl-L-cysteine (NAC) or were subjected to a knock-down of the AhR gene, after which they were treated with the selected PM concentrations for 2 hours. AhR knock-down was confirmed by suppression of the mRNA expression levels of AhR (Figure 3A). As shown in Figure 3B and Figure S1, the mRNA expression levels of IL1-alpha and IL1-beta were significantly decreased following AhR knock-down, whereas NAC had no statistically significant inhibitory effect on the mRNA expression levels of either IL1-alpha or IL1-beta. However, the protein expression levels of IL1-alpha were not significantly reduced, whereas IL1-beta protein levels...
were suppressed in HaCaT cells with an AhR knock-down. For the experiment of the role of AhR and NAC on the indirect MMP1 and COX2 regulation in HDF which indirectly activated by PM. A, AhR gene was knocked-down (K/D) by its specific siRNA with lipofectamine3000. B, The mRNA and protein expression levels of IL1-alpha and -beta was analysed when AhR gene K/D on HaCaT cells. C, The protein and mRNA expression levels of MMP1 and the mRNA levels of COX2 on HDF which was co-cultured with HaCaT cells were also conducted of the AhR gene K/D. The relative mRNA levels were presented as fold change compared with none treated cells (0 μg/cm²) Data are presented as the mean ± SEM of three independent experiments (n = 3). *, P < 0.05, **, P < 0.005 vs NC. #, P < 0.05, vs pmA-treated group. &, P < 0.05, vs pmB-treated group. NS, no significance.

**FIGURE 3** The role of AhR both on induction of IL1-alpha and -beta in PM-treated HaCaT and regulation of MMP1 and COX2 in HDF which indirectly activated by PM. A, AhR gene was knocked-down (K/D) by its specific siRNA with lipofectamine3000. B, The mRNA and protein expression levels of IL1-alpha and -beta was analysed when AhR gene K/D on HaCaT cells. C, The protein and mRNA expression levels of MMP1 and the mRNA levels of COX2 on HDF which was co-cultured with HaCaT cells were also conducted of the AhR gene K/D. The relative mRNA levels were presented as fold change compared with none treated cells (0 μg/cm²) Data are presented as the mean ± SEM of three independent experiments (n = 3). *, P < 0.05, **, P < 0.005 vs NC. #, P < 0.05, vs pmA-treated group. &, P < 0.05, vs pmB-treated group. NS, no significance.

**FIGURE 4** Particulate matter (PM)-treated HaCaT-derived IL1-alpha and IL1-beta produced by via p38 kinase activation induced the expression of MMP1 and COX2 expression in fibroblasts. A, c-Jun and c-Fos proteins, components of the AP-1 transcription factor, were phosphorylated after PM treatment. The specific inhibitor of p38 kinase (SB203580) attenuated the phosphorylation of B, p38 MAPK, c-Fos and c-Jun phosphorylation. HaCaT cells were treated with SB203580 (SB) inhibitor before PM treated. The protein expression levels were analysed by Western blot analysis. C, the IL1-alpha and -beta expression was measured using the conditioned media by ELISA assays. D, the phosphorylated H2AX protein on HDF after co-cultured with PM-treated HaCaT cells (the relative expression levels were quantified and presented in graphical form). The HDF were co-cultured with HaCaT cells which was treated with SB 1 h before PM and the cells were incubated for 24 h. The protein expression levels were analysed by Western blot analysis. E, The mRNA and protein expression levels of MMP1 and COX2 on HDF which was co-cultured with PM-treated HaCaT cells were assessed by real-time PCR and ELISA assay, respectively. The COX2 expression levels of mRNA and protein were assessed by real-time PCR and Western blot analysis, respectively. Data are presented as the mean ± SEM of three independent experiments (n = 3). *, P < 0.05, **, P < 0.005 vs NC. #, P < 0.05, vs pmA-treated group. &, P < 0.05, vs pmB-treated group. NS, no significance.
3.4 | IL1-alpha and IL1-beta induction in PM-treated HaCaT cells were mainly regulated via p38 kinase-dependent pathways

As described previously, the transcriptional regulation of the IL1-alpha and IL1-beta genes is mediated by activator protein 1 (AP-1) which includes c-Jun and c-Fos. [17,18] Figure 4A shows that both the c-Jun and c-Fos proteins, components of the AP-1 transcription factor, were phosphorylated after PM treatment. To determine whether this effect was mediated by p38 signalling, a p38 inhibitor SB203580 (10 μmol/L) was used to treat cells 1 hour before PM treatment. As shown in Figure 4, the phosphorylation of c-Jun and c-Fos was effectively attenuated by the p38 inhibitor treatment. In addition, the levels of IL1-alpha and IL1-beta, as assessed by ELISA, were markedly decreased by treatment with the p38 inhibitor (Figure 4C).

3.5 | Keratinocyte-derived IL1-alpha and IL1-beta produced by p38 kinase activation induce the expression of MMP1 and COX2 in fibroblasts

The p38 inhibitor SB203580 (10 μmol/L) was used to treat keratinocytes, and the expression levels of MMP1 and COX2 in fibroblasts were then assessed. Cells were pretreated for 1 hour before PM treatment. The insert containing the SB203580-treated HaCaT keratinocytes was moved to the fibroblast culture plate, and the HaCaT cells were treated with PM for 24 hours. The protein and mRNA expression of levels of MMP1 and COX2 were significantly attenuated in the fibroblasts co-cultured with SB203580-treated HaCaT keratinocytes (Figure 4E). Moreover, the protein expression of H2AX, which is a marker of DNA damage, was decreased under the same conditions used in Figure 4D.

4 | DISCUSSION

Urban PM is more toxic to skin cells when it contains environmental hormones, incomplete combustion products and heavy metals. Therefore, standard reference materials (SRM) issued by the National Institute of Standards and Technology (NIST) were used for this study. One of these was SRM 1648a (pmA) which was used as a NIST characteristic of urban particulate matter mainly containing heavy metals, and the other was SRM 1649b (pmB) which was used as a NIST characteristic of urban dust mainly containing polycyclic aromatic hydrocarbons (PAHs). As shown in Figures 1 and 4, even at concentrations that were not cytotoxic, there was an induction of an inflammatory response in epidermal cells, and there was DNA damage to the dermal cells. It also has a bigger impact when environmental hormones are involved than when it contains heavy metal.

The present study was undertaken to investigate the mechanisms of PM effects on epidermal inflammation and skin ageing using an in vitro co-culture model of human keratinocytes and fibroblasts. As is well known, PM caused adverse inflammatory responses in HaCaT cells by (a) AhR activation, (b) ROS generation, and (c) phosphorylation of p38 MAPKs (Figure 1). Moreover, the data from the analysis of the pro-inflammatory responses in HaCaT cells showed that there was secretion of inflammatory cytokines including IL1-alpha, IL1-beta, IL6 and IL8 (Figure 2C,D). Therefore, the possibility that there was a paracrine effect of these cytokines to regulate the degradation of dermal collagen through an indirect effect of PM on dermal fibroblasts was explored using an in vitro co-culture model of HaCaT keratinocytes and HDF fibroblast cells. To explore this hypothesis, recombinant IL1-alpha, IL1-beta, IL6 and IL8 were used to treat HDF cells. As shown in Figure 2, IL1-alpha and IL1-beta significantly regulated the expression of MMP1 and COX2 in HDF cells. From the results, we hypothesized that PM-induced, keratinocyte-derived IL1-alpha and IL1-beta would act to increase the levels of MMP1 and COX2 in fibroblasts. In human skin, MMP1 is the major protease capable of initiating the fragmentation of native fibrillar collagens, predominantly type I and III collagens. Once cleaved by MMP1, collagen can be further degraded by both MMP-3 and MMP-9. Dermal collagen fibrils are stabilized by cross links that are highly resistant to proteolytic cleavage. Therefore, the actions of elevated MMPs over decades are thought to result in the accumulation of cross-linked collagen fragments, which impair the structure and function of the dermal ECM. Indeed, senescent fibroblasts are characterized by high MMP secretion levels. [20] To explore the molecular mechanisms underlying the induction of IL1-alpha and IL1-beta in HaCaT cells, the three types of PM-induced inflammatory responses (AhR activation; ROS generation; phosphorylation of p38 MAPKs) were inhibited through the use of specific inhibitors or siRNA as appropriate. As shown in Figures 3 and 4, the mRNA expression levels of IL1-alpha and IL1-beta were significantly decreased by p38 inhibitor treatment, whereas AhR knock-down only suppressed IL1-alpha expression; NAC had no statistically significant effect on both cytokines (Figures S1 and S2). Furthermore, NOX inhibitors or ROS scavengers also did not have any effect on the regulation of the expression of IL1-alpha and IL1-beta (data not shown). A previous study by [16] has shown that AhR activation results in ROS generation and p38 kinase phosphorylation. However, there was no evidence in our study of a direct relationship between AhR activation and p38 kinase activity. We assume that they separately regulate the expression of IL1-alpha or IL1-beta.

As the skin plays a role as the primary immunological barrier to the external environment, the innate immune system provides defense against external stimuli via innate immune receptors and the release of pro-inflammatory cytokines. [21] There is a strong association of ageing with chronic low-grade inflammatory activity which may progress to long-term tissue damage and chronic systemic inflammation. Accumulating evidence supports the concept of “inflammaging”, and inflammation and ROS accumulation are now believed to be the causative factors in skin ageing. An inflammatory state characterized by an increase in pro-inflammatory markers, including TNF-α, IL6, IL1-beta and C-reactive protein, is believed to contribute to, or worsen, a general decline in the biological mechanisms responsible for physical function during ageing. [22]

The IL1 cytokine family consists of 11 members, and many of these are potent pro-inflammatory agents (IL1-alpha, IL1-beta and IL-18) allowing innate immune receptors to trigger signals
protection against infection or injury. [24] Recent reports have suggested that keratinocyte-derived IL1 stimulates collagen turnover in the dermis through a tight regulation of both collagen and collagenase synthesis in fibroblasts. [10,25] Moreover, IL1-beta-induced PGE2 formation is mediated by an enhanced expression of COX2 in human gingival fibroblasts. [25] This suggests that the PM-induced excessive secretion of IL1-alpha and IL1-beta from epithelial keratinocytes contributes to triggering collagen degradation mechanisms in fibroblasts. In addition, the DNA damage marker histone H2AX was markedly attenuated in fibroblasts co-cultured with p38 inhibitor-treated HaCaT cells. The histone H2AX has been identified as a component in DNA repair mechanisms and as an indicator of oxidative cell stress in epidermal cells.

These results demonstrate that the increased expression of MMP1 and COX2 in fibroblasts, resulting from keratinocyte-derived IL1-alpha and IL1-beta secretion, mediates dermal collagen degradation via a p38 kinase-dependent pathway in the keratinocytes. These findings support a novel mechanism for PM-induced dermal collagen disruption and the formation of wrinkles in skin that is controlled by p38 kinase. We suggest that the regulation of p38 kinase can be useful for finding candidate agents to improve PM-induced skin ageing in the future.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

MJK involved in conceptualization, data curation, formal analysis, investigation, visualization and writing – review and editing. JHK involved in data curation, investigation, visualization and writing – review and editing. GJJ, MD, involved in investigation and formal analysis. KYP, MD, PhD, involved in formal analysis, supervision and writing – review and editing. M-KL, MD, PhD, involved in investigation, data curation and supervision. SJS, MD, PhD, involved in conceptualization, supervision and writing – review and editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The mRNA expression levels of IL1 alpha and beta was analyzed when NAC treated condition on HaCaT cells

Figure S2. The mRNA expression levels of MMP1 and COX2 on HDF which was co cultured with HaCaT cells which were NAC treated

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