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Kaempferol impedes IL-32-induced monocyte-macrophage differentiation

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ABSTRACT

Kaempferol possesses a wide range of therapeutic properties, including antioxidant, anti-inflammatory, and anticancer properties. The present study sought to evaluate the effects and possible pharmacological mechanisms of kaempferol on interleukin (IL)-32-induced monocyte-macrophage differentiation. In this study, we performed flow cytometry assay, immunocytochemical staining, quantitative real-time PCR, enzyme-linked immuno sorbent assay, caspase-1 assay, and Western blotting to observe the effects and underlying mechanisms of kaempferol using the human monocyte cell line THP-1. The flow cytometry, immunocytochemical staining, and real-time PCR results show that kaempferol attenuated IL-32-induced monocyte differentiation to product macrophage-like cells. Kaempferol decreased the production and mRNA expression of pro-inflammatory cytokines, in this case thymic stromal lymphopoietin (TSLP), IL-1β, tumor necrosis factor (TNF)-α, and IL-8. Furthermore, kaempferol inhibited the IL-32-induced activation of p38 and nuclear factor-κB in a dose-dependent manner in THP-1 cells. Kaempferol also ameliorated the lipopolysaccharide-induced production of the inflammatory mediators TSLP, IL-1β, TNF-α, IL-8, and nitric oxide of macrophage-like cells differentiated by IL-32. In brief, our findings may provide new mechanistic insights into the anti-inflammatory effects of kaempferol.

1. Introduction

Inflammation is an important biological defense response against various stimuli, including pathogens, damaged cells, and irritants. The inflammatory response is beneficial to remove pathogens and promotes (repair) wound healing, but a dysregulated inflammatory response leads to progressive tissue destruction and chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, autoimmune disease, diabetes, Alzheimer's disease, and even certain types of cancer [1,2]. The pathogenesis of inflammation requires an increase in the number of priming inflammatory immune cells producing mediators and cytokines [3]. Monocytes are well known to an cause inflammatory response by inducing the production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), thymic stromal lymphopoietin (TSLP), interleukin (IL)-1β, IL-8, and IL-6 [4,5]. Circulating monocytes are recruited to sites of inflamed tissue where, following conditioning by the local growth factors, pro-inflammatory cytokines, and microbial products, they differentiate into macrophages [6]. Macrophages also play a key role in inflammatory immune responses by releasing inflammatory cytokines such as TNF-α and IL-6, as well as mediators, including nitric oxide (NO) [7].

IL-32 is a major proinflammatory cytokine that causes diverse types of inflammation, such as Crohn's disease, rheumatoid arthritis, chronic obstructive pulmonary disease, ulcerative colitis, psoriasis, atopic dermatitis, and tuberculosis [8–13]. IL-32 is expressed by T cells, NK cells, keratinocytes, and fibroblasts [14,15]. IL-32 is able to stimulate inflammatory cytokine production in various cell types [16]. Netea and coworkers reported that IL-32 is a key factor for the differentiation of monocytes into macrophage-like cells [16].

Beauty-salt™ (Bongsunhwa Food, Kwangju, Korea) consists of solar salt and *Impatiens balsamina* L. extract and has been used to treat patients with various inflammatory disorders in Korea. Kaempferol (3,5,7-trihydroxy-2-(-4-hydroxyphenyl)-4H-1-benzopyran-4-one), a natural and active flavonoid compound [17,18], is a major compound of Beauty-salt™. Numerous previous

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studies have demonstrated that kaempferol possesses a wide range of therapeutic properties, including antioxidant, anti-inflammatory and anticancer properties [17]. In recent work, we showed a potent anti-allergic effect of kaempferol in on eosinophil-derived inflammatory responses [19]. However, there have been no reports on the action of kaempferol against monocytes-mediated inflammation. In the present study, we aim to evaluate the effects of kaempferol on IL-32-induced monocyte differentiation and inflammatory responses.

2. Materials and methods

2.1. Reagents

We purchased kaempferol (purity; > 97%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bicinchoninic acid, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), *E. coli* lipopolysaccharide (LPS), and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA); human recombinant IL-32, human recombinant TSLP and IL-1β, anti-human TSLP and IL-1β antibodies (Ab), and biotinylated anti-human TSLP and IL-1β Ab from R&D Systems (Minneapolis, MN, USA); human recombinant TNF-α, IL-6, and IL-8, anti-human TNF-α, IL-6, and IL-8 Ab, and biotinylated anti-human TNF-α, IL-6, and IL-8 Ab from BD Biosciences Pharmingen (San Diego, CA); NF-κB, actin, p38, phosphorylated p38 (pp38), poly (ADP-ribose) polymerase (PARP), caspase-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ab from Santa Cruz Biotechnology (Santa Cruz, CA, USA); CD11b and CD14 Ab from eBioscience (San Diego, CA, USA); CD44 antibody from abcam (Cambridge, MA, USA).

2.2. Cells

THP-1 human monocyte cells obtained from the American Type Culture Collection (TIB-202; Manassa, VA, USA) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere in the presence of 5% CO_2. Kaempferol was prepared by dissolving with DMSO and diluted with RPMI 1640 medium.

2.3. Flow cytometry

THP-1 cultured in the presence or absence of IL-32 and kaempferol for 6 days were washed in fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline supplemented with 1% bovine serum albumin and 0.1% NaN) and then incubated with primary Ab at room temperature for 30 min. The primary Ab were phycoerythrin (PE)-conjugated anti-human CD11b and CD44 or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14. After washing with FACS buffer, cells were fixed with 1% (weight/volume) paraformaldehyde for 30 min and then stored in the dark until analyzed by flow cytometry. Cytofluorometry was performed with a FACScan (Becton Dickinson, Mountain View, CA, USA).

2.4. Analysis of macrophage surface markers by confocal laser scanning microscopy

THP-1 cells cultured in the presence or absence of IL-32 and kaempferol for 6 days were fixed with 4% formaldehyde. After washed in PBS, cells were blocked with bovine serum albumin followed by 60 min of incubation with 2 μl phycoerythrin (PE)-conjugated anti-human CD11b and CD44 or fluorescein isothiocyanate (FITC)-conjugated anti-human CD14. The mounting medium containing 4′, 6-diamidino-2-phenylinodole (DAPI, Vector Laboratories, Burlingame, CA, USA) was used to counterstain DNA. All specimens were examined with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.5. Quantitative real-time PCR analysis

Quantitative real-time PCR was performed using a SYBR Green Master Mix and the detection of mRNA was analyzed using an ABI StepOne Real-time PCR System (Applied Biosystems, foster City, CA, USA). Primer sequences for the reference gene GAPDH and the genes of interest were as follows: GAPDH (5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′; 5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′); human TSLP (5′-CCC AGG CTA TTC GGA AAC TCA G-3′; 5′-CGC CAC AAT CCT TGT AAT TGT G-3′); human IL-1β (5′-AAA CAG ATG AAG TGC TCC TT-3′; 5′-TGG AGA ACA CCA CTT GTT GC-3′); human TNF-α (5′-AGG ACG AAC ATC CAA CCT TCC CAA-3′; 5′-TTT GAG CCA GAA GAG GTT GAG GGT-3′); human IL-8 (5′-CGA TGT CAG TGC ATA AAG ACA-3′; 5′-TGA ATT CTC AGC CCT CTT CAA AAA-3′); human CD11b (5′-ACG TAA ATG GGA CAA GCT G-3′; 5′-GAT CCT GAG GTT CCG TGA AA-3′); human CD14 (5′-ACT TGC ACT TTC CAG CTT GC-3′; 5′-GCC CAG TCC AGG ATT GTC AG-3′). Typical profile times were the initial step, 95 °C for 10 min followed by a second step at 95 °C for 15 s and 60 °C for 30 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the GAPDH and compared with the control. Data were analyzed using the ΔΔCT method.

2.6. MTT assay

Cell viability was determined by a MTT assay. Briefly, 100 μl of cells suspension (1×10^4 cells) was cultured in 96-well plates after pretreatment with kaempferol. The MTT solution (5 mg/ml) was added and the cells were incubated at 37 °C for 4 h. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, the optical density was measured using an ELISA reader at 540 nm.

2.7. Sandwich enzyme-linked immunosorbent assay (ELISA)

The amounts of $_{TSLP,\ IL\text{-}1\beta}$, TNF-$_{\alpha,\ IL\text{-}6,\ and\ IL\text{-}8}$ in supernatants were measured using a sandwich ELISA method. Briefly, 96-well plates were coated with 100 μl aliquots of anti-human $_{TSLP,\ IL\text{-}1\beta}$, TNF-$_{\alpha,\ IL\text{-}6,\ and\ IL\text{-}8}$ Ab and incubated overnight at 4 °C. After additional washes, 100 μl of a cell medium or recombinant $_{TSLP,\ IL\text{-}1\beta}$, TNF-$_{\alpha,\ IL\text{-}6,\ and\ IL\text{-}8}$ standards were added and incubated at 37 °C for 2 h or 4 h. After the wells were washed, biotinylated anti-human $_{TSLP,\ IL\text{-}1\beta}$, TNF-$_{\alpha,\ IL\text{-}6,\ and\ IL\text{-}8}$ Ab were added and incubated at 37 °C for an additional 2 h. Subsequently, the wells were washed, avidin-peroxidase was added, and theses samples were incubated for 30 min at 37 °C. After the wells were washed, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) as a substrate was added. Color development at 405 nm was then measured using an ELISA reader. In addition, a standard curve was generated for each assay plate by measuring the absorbance of serial dilutions of recombinant $_{TSLP,\ IL\text{-}1\beta}$, TNF-$_{\alpha,\ IL\text{-}6,\ and\ IL\text{-}8}$ at 405 nm. All samples were performed in duplicate.

2.8. Caspase-1 enzymatic activity assay

Caspase-1 enzymatic activity was measured according to the manufacturer's specification by using a caspase assay kit (R&D Systems).

2.9. Western blot analysis

The stimulated cells were lysed and separated through 10% SDS-PAGE. After electrophoresis, the protein was transferred to