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Rosmarinic acid inhibits poly(I:C)-induced inflammatory reaction of epidermal keratinocytes

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ABSTRACT

Aims: Keratinocytes are the predominant cells in the epidermis, exerting their primary role of physical barrier through sophisticated differentiation process. In addition, keratinocytes contribute to the activation of innate immunity, providing the surveillant role against external pathogens. It has been known that chronic skin inflammatory disease such as psoriasis can be provoked by viral pathogens including double-stranded RNA. In this study, we demonstrated that rosmarinic acid (RA) has an inhibitory potential on inflammatory reaction induced by double-stranded RNA mimic poly(I:C) in epidermal keratinocytes.
Main methods: We cultured human epidermal keratinocytes and induced inflammatory reaction by poly(I:C) treatment. The effect of RA on inflammatory reaction of keratinocytes was determined by RT-PCR and Western blot.
Key findings: RA significantly inhibited poly(I:C)-induced expression of inflammatory cytokines including IL-1β, IL-6, IL-8, CCL20, and TNF-α, and downregulated NF-κB signaling pathway in human keratinocytes. In addition, RA significantly inhibited poly(I:C)-induced inflammasome activation, in terms of secretion of active form of IL-1β and caspase-1. Furthermore, RA markedly inhibited poly(I:C)-induced NLRP3 and ASC expression.
Significance: These results indicate that RA can inhibit poly(I:C)-induced inflammatory reaction of keratinocytes, and suggest that it may be a potential candidate for the treatment of psoriasis.

1. Introduction

Skin is the largest organ, encompassing the entire body and demarcating the organism and environment. Its main function is to protect the body against harmful external insults [1]. The outmost layer epidermis is comprised of several cell types including keratinocytes, melanocytes and Langerhans cells. Among those, keratinocytes are the predominant cells and exert their role for physical barrier against external stimuli. In addition, keratinocytes contribute to innate immune surveillance through expressing various Toll-like receptors (TLRs), kinds of important pattern recognition receptors (PRRs). The specific recognition of pathogen-associated molecular patterns (PAMPs) by keratinocytes results in activation of inflammation-related intracellular signaling and production of inflammatory cytokines [2,3].

Psoriasis is an immune-mediated inflammatory skin disorder characterized by hyperplasia of epidermal keratinocytes and infiltration of inflammatory cells [4]. It can be provoked or exacerbated by risk factors affecting the skin barrier function such as infections and mild trauma (scratching, piercings), suggesting that keratinocytes may be the potential origin cells for psoriasis. The cross-talk between innate and adaptive immunity is the fundamental mechanistic basis for psoriasis, in which many cytokines are functionally involved [5]. Especially, stimulation of keratinocytes with various PAMPs results in activation of innate immunity of keratinocytes, leading to the production of inflammatory cytokines related with psoriasis, including IL-1β and TNF-α [6]. Considering the importance of this initial innate immune response, blocking the inflammatory reaction of keratinocytes can be a good target for treating psoriasis.

Polyinosinic:polycytidylic acid (poly(I:C)) is a synthetic analogue of viral double-stranded RNA (dsRNA) which can induce innate immune response in a TLR3-dependent manner [7]. Keratinocytes express high level of TLR3, and stimulation of this receptor with its synthetic ligand poly(I:C) provokes inflammatory reaction in epidermal keratinocytes [8,9]. In an attempt to find the therapeutics for psoriasis, we found that rosmarinic acid (RA) can be a possible candidate for drug development. It has been reported that RA possesses antioxidant and anti-inflammatory properties in macrophages [10]. However, the effect of RA

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on epidermal keratinocytes remains to be elucidated. In this study, we demonstrated that RA has an inhibitory potential on poly(I:C)-induced inflammatory reaction of epidermal keratinocytes.

2. Materials and methods

2.1. Cell culture and drug preparations

Human skin tissues were obtained under the written informed consent of donors, in accordance with the ethical committee approval process of the Institutional Review Board of Chungnam National University Hospital (IRB file number: 2015-05-013). Primary keratinocytes were cultured according to the method previously described. Primary keratinocytes were isolated from epidermis, and then immortalized using the recombinant retrovirus expressing simian virus 40 T antigen (SV40Tag) [11]. SV40Tag-transformed human epidermal keratinocytes (SV-HEKs) were routinely cultured in keratinocyte-serum free medium (K-SFM) supplemented with bovine pituitary extract (BPE) and recombinant human epidermal growth factor (rhEGF) (Life Technologies Corporation, Grand Island, NY). For treatment with poly(I:C), culture medium was changed into MCDB153 (Biochrom GmbH, Berlin, Germany) supplemented with BPE and rhEGF, and cells were incubated overnight. Then, cells were replenished with fresh MCDB153 and poly(I:C) (InvivoGene, San Diego, CA, USA) was added at the final concentration of 1 μg/ml. Rosmarinic acid (RA) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO).

2.2. Cytotoxicity test

SV-HEKs were seeded in 24-well culture plate at a density of 1×10^5/well, treated with RA for 24 h. Then the medium was replaced with fresh medium containing 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution and cells were incubated for an additional 4 h. Finally, formazan crystal was dissolved with DMSO. Cell viability was determined by measuring optical density at 570 nm using an ELISA reader. For lactate dehydrogenase (LDH) assay, culture medium was collected 24 h after treatment with RA. Enzyme activity was determined using Cytotoxicity detection kit (Roche, Indianapolis, IN), according to the manufacturer's protocol.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Easy-blue RNA extraction kit (Intron, Daejeon, Korea). For qRT-PCR, approximately 2 μg of total RNAs were reverse transcribed with moloney-murine leukaemia virus (M-MLV) reverse transcriptase (RTase) (Elpis Biotech, Daejeon, Korea). The equal volume of cDNAs was then used as templates for PCR reaction with SYBR Green Master Mix (Applied Biosystems, Warrington, UK). The qRT-PCR was performed using ABI StepOne Real-Time PCR system (Applied Biosystems). The gene-specific primers were used as below: IL-1β, 5′-TTAAAGCCCGCCTGACAGA and 5′-GCGAATGACAGAGGGTTTCTTAG; IL-6, 5′-CTGCGCAGCTTTAAGGAGTTC and 5′-CCATGCTACATTTGCC GAAGA; IL-8, 5′-CCTTTCCACCCCAAATTTATCA and 5′-TTTCTGTGTTG GCGCAGTGT; CCL20, 5′-CCACCTCTGCGGCGAAT and 5′-TGTGTATC CAAGACAGCAGTCAAA; TNF-α, 5′-CTCCTTCAGACACCCTCAACCT and 5′-CGACCCTAAGCCCCCAATT; GAPDH, 5′-TGCACCACCAACTGCTTAGC and 5′-GGCATGGACTGTGGTCATGAG.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Culture medium was collected, and protein levels of secreted IL-1β, IL-8 and TNF-α were determined using commercial ELISA kits. IL-8 kit was purchased from Life Technologies Corporation (Grand Island, NY). IL-1β and TNF-α kits were purchased from R&D Systems (Minneapolis, MN).

2.5. Western blotting

SV-HEKs were lysed with Proprep solution (Intron, Daejeon, Korea). Protein concentrations were measured using BCA protein assay kit (Pierce, Rockford, IL). Samples were run on SDS-polyacrylamide gels, transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in Tris-buffered saline plus Tween 20 (TBS-T), membranes were incubated with appropriate primary antibodies, and then incubated with horseradish peroxide-conjugated secondary antibodies, visualized by enhanced chemiluminescence (Intron, Daejeon, Korea). For detection of secreted proteins, culture medium was concentrated using a Protein concentration kit (Elpis Biotech, Daejeon, Korea). The following primary antibodies were used: phospho-p65, phospho-IκBα, caspase-1 (Cell Signaling Technology, Beverly, MA); IL-1β (Abcam, Cambridge, MA); NLRP3 and ASC (AdipoGen, San Diego, CA); actin (Sigma-Aldrich, St. Louis, MO).

2.6. NF-κB reporter assay

Keratinocytes were seeded in 12-well plate, then transduced with NF-κB reporter adenovirus for overnight [12]. Cells were replenished with fresh medium and incubated for a further 24 h. Cells were then treated with poly(I:C) and/or RA for 24 h, and luciferase activity was measured using dual luciferase reporter assay system (Promega, Madison, WI).

2.7. Statistical analysis

All data were derived from at least three independent experiments. The results were presented as mean ± standard deviation (SD). Data were evaluated statistically by one-way ANOVA or Student's *t*-test using SPSS software v 22.0 (IBM, Seoul, Korea). Statistical significance was set at $P < 0.01$.

3. Results

3.1. Cytotoxicity of rosmarinic acid (RA) on keratinocytes

We tried to seek potential therapeutics for psoriasis, and found that rosmarinic acid (RA) (Fig. 1A) has inhibitory potential on inflammatory reaction of human keratinocytes. At first, we determined the cytotoxicity of RA on SV-HEKs. The MTT assay showed that RA did not induce cell death up to the dose of 100 μM (Fig. 1B). In LDH assay, the doses over 50 μM resulted in increased secretion of LDH from SV-HEKs (Fig. 1C). These results implicated that RA caused some damage of plasma membrane. Therefore, we treated keratinocytes with RA up to 20 μM in next experiments.

3.2. The effect of rosmarinic acid (RA) on poly(I:C)-induced cytokines release

We examined the effect of RA on poly(I:C)-induced immune response of keratinocytes using qPCR and ELISA. After treatment with poly(I:C), the mRNA levels for inflammatory cytokines including IL-1β, IL-6, IL-8, CCL20 and TNF-α increased dramatically, while RA pretreatment significantly inhibited the poly(I:C)-induced cytokines expression (Fig.2A). Consistent with these results, poly(I:C) increased secretion of IL-1β, TNF-α and IL-8 from keratinocytes, and RA pretreatment significantly inhibited poly(I:C)-induced cytokines release (Fig. 2B).

To determine whether RA can reduce poly(I:C)-mediated molecular machine of inflammation after its onset, we performed experiments in which RA was treated after poly(I:C) stimulation. As shown in Supplement 1A, RA treatment 5 min after poly(I:C) stimulation resulted in significant inhibition of poly(I:C)-induced cytokine expression. In contrast, RA treatment 30 min after poly(I:C) stimulation failed to inhibit