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Original Article

Inhibitory effect of Paeonia lactiflora Pallas extract (PE) on poly (I:C)-induced immune response of epidermal keratinocytes

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Abstract: Epidermal keratinocytes provide protective role against external stimuli by barrier formation. In addition, kertinocytes exerts their role as the defense cells via activation of innate immunity. Disturbance of keratinocyte functions is related with skin disorders. Psoriasis is a common skin disease related with inflammatory reaction in epidermal cells. We attempted to find therapeutics for psoriasis, and found that Paeonia lactiflora Pallas extract (PE) has an inhibitory potential on poly (I:C)-induced inflammation of keratinocytes. PE significantly inhibited poly (I:C)-induced expression of crucial psoriatic cytokines, such as IL-6, IL-8, CCL20 and TNF-α, via down-regulation of NF-κB signaling pathway in human keratinocytes. In addition, PE significantly inhibited poly (I:C)-induced inflammasome activation, in terms of IL-1β and caspase-1 secretion. Finally, PE markedly inhibited poly (I:C)-increased NLRP3, an important component of inflammasome. These results indicate that PE has an inhibitory effect on poly (I:C)-induced inflammatory reaction of keratinocytes, suggesting that PE can be developed for the treatment of psoriasis.

Keywords: Keratinocytes, poly (I:C), Paeonia lactiflora Pallas extract, NF-κB, inflammasome

Introduction

Skin is the outmost organ that provides protective barrier against environmental insults such as microbial infection, chemicals and ultraviolet (UV) radiation [1]. Skin is comprised of specified three layers including epidermis, dermis, and subcutaneous layer. Among many cell types comprising the skin, three types of cells including keratinocytes, melanocytes and fibroblasts are regarded as the major cells to maintain the structural basis and homeostasis. Particularly, epidermal keratinocytes make the rigid water-insoluble structure called cornified cell envelope (CE) through the sophisticated differentiation program, thereby contributing to establishing protective barrier [2, 3]. In addition to their essential role for the building block of physical barrier, keratinocytes exert their important role as the primary defense cells. Keratinocytes express a range of Toll-like receptors (TLRs), the pattern recognition receptors (PRRs) in human innate immunity. The recognition of bacterial pathogen-associated molecular patterns (PAMPs) by keratinocytes led to activation of inflammation-related intracellular signaling and production of inflammatory cytokines from keratinocytes [4-6].

Psoriasis is a chronic inflammatory skin disease. The incidence of psoriasis is relatively high, reaching 0.5-3% of the population worldwide. The characteristic features of psoriasis include keratinocyte hyperproliferation, altered keratinocyte differentiation, and inflammation [7]. It has long been notified that psoriasis is a Th1-type immune cell-mediated disease, because that immunosuppressant cyclosporine A (CsA) efficiently blocks the activation of T cells and consequently improves the psoriatic symptoms [8]. Additionally, recent investigations emphasize the important role of keratinocytes in the pathophysiology of psoriasis. For example, psoriasis can be triggered and/or exacer-

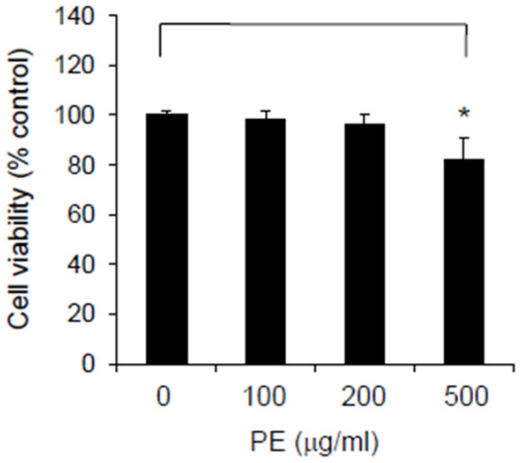

Figure 1. Cytotoxicity of Paeonia lactiflora Pallas extract (PE). SV40Tag-transformed human epidermal keratinocytes (SV-HEKs) were treated with PE at the indicated concentrations for 24 h. Cell viability was measured by MTT assay. The mean values ± SD are averages of triplicate measurements. $^{*}P<0.01$.

bated by physical trauma on the skin, suggesting that keratinocytes may be the potential origin cells for psoriasis. Stimulation of keratinocytes with various PAMPs or damage-associated molecular patterns (DAMPs) results in activation of innate immunity, leading to production of inflammatory cytokines related with psoriasis [9-11]. In this regard, the inflammatory reaction of keratinocytes can be a good target for the development of novel therapeutics on psoriasis.

Polyinosinic: polycytidylic acid (poly (I:C)) is an immunostimulant that activates TLR3. The structure of poly (I:C) resembles double-stranded RNA present in some viruses [12]. Poly (I:C) is widely used for studying the innate immunity-related skin diseases such as psoriasis [10]. We attempted to find therapeutics on psoriasis using poly (I:C)-induced inflammation model, and found that Paeonia lactiflora Pallas extract (PE) has an inhibitory potential on inflammatory reaction. Paeonia lactiflora Pallas has long been used for the treatment of inflammatory disorders in oriental medicine [13]. However, the effect of PE on inflammatory reaction in epidermal keratinocytes remains to be elucidated. In this study, we demonstrate that PE inhibits poly (I:C)-induced inflammatory reaction in keratinocytes, suggesting that PE can be applicable for psoriasis treatment.

Materials and methods

Cell culture

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. Primary keratinocytes were cultured according to the method previously described [14]. For immortalization, keratinocytes were transduced with the recombinant retrovirus expressing simian virus 40 T antigen (SV40Tag) and selected using G418 for 4 weeks [15]. 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).

Preparation of Paeonia lactiflora Pallas extract (PE)

The air-dried root of Paeonia lactiflora Pallas (Omniherb Co, Daegu, Korea) was cut into pieces and extracted with 8 volumes of boiling-water for 4 h. The extract was filtered using Whatman paper, evaporated under reduced pressure condition, and then freeze-dried (Eyela, Irvine, CA). For treatment of cultured cells, PE was reconstituted in distilled water and filter-sterilized.

Cell viability test

SV-HEKs were seeded in 6-well plate at a density of 2×10^5, treated with PE for 24 h. After treatment, cells received 2 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution and were incubated for a further 4 h. Cell viability was determined by measuring optical density at 540 nm using an ELISA reader.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNAs were isolated using Easy-blue RNA extraction kit (Intron, Daejeon, Korea). Two μg of total RNAs were reverse transcribed with moloney-murine leukaemia virus (M-MLV)

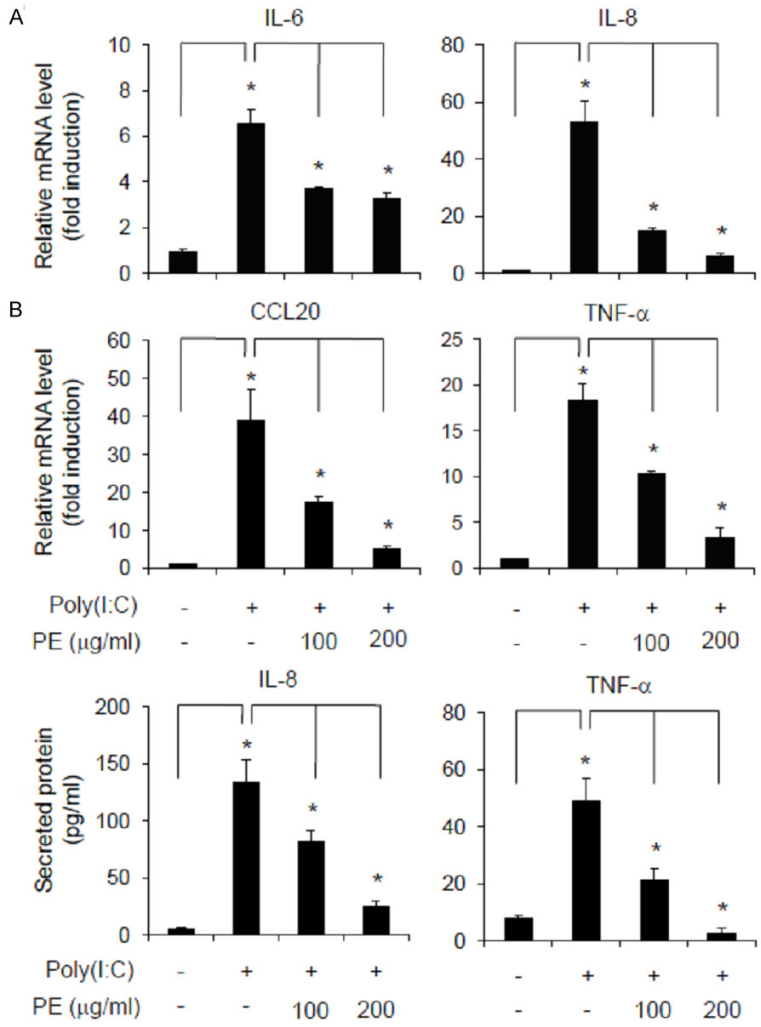

Figure 2. Effect of PE on poly (I:C)-induced inflammatory reaction in keratinocytes. A. SV-HKEs were pretreated with PE at the indicated concentrations for 1 h, and then stimulated with 1 μg/ml poly (I:C) for 2 h. The mRNA level was determined by qPCR. Data are expressed as fold induction. B. SV-HKEs were pretreated with PE for 1 h, and then stimulated with 1 μg/ml poly (I:C) for 24 h. Released cytokines were measured by ELISA. The mean values ± SD are averages of triplicate measurements. *P<0.01.

reverse transcriptase (RTase) (Elpis Biotech, Daejeon, Korea). Aliquots of RT mixture were amplified using SYBR Green real-time PCR master mix (Elpis Biotech). The following primers sequences were used: IL-6, 5'-CTGCGCAGCTTTAAGGAGTTC and 5'-CCATGCTACATTTGCCGAAGA; IL-8, 5'-CCTTTCCACCCCAAATTTATCA and 5'-TTTCTGTGTTGGCGCAGTGT; CCL20, 5'-CCACCTCTGCGGCGAAT and 5'-TGTGTATCCAAGACAGCAGTCAAA; TNF-α, 5'-CTCCTTCAGACACCCTCAACCT and 5'-CGACCCTAAGCCCCCAATT; GAPDH, 5'-TGCACCACCAACTGCTTAGC and 5'-GGCATGGACTGTGGTCATGAG.

ELISA

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

Western blotting

Cells were lysed in Proprep solution (Intron, Daejeon, Korea). Total protein was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Samples were run on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated with appropriate antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies, visualized by enhanced chemiluminescence (Intron). For determination of secreted proteins, cell culture medium was concentrated using a Protein concentration kit (Elpis Biotech). The following primary antibodies were used in this study: 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).

Statistical analysis

Data were evaluated statistically using one-way analysis of variance (ANOVA) with the SPSS software (v 22.0; IBM, Seoul, Korea). Statistical significance was set at P<0.01.

Results

We attempted to find potential therapeutics for psoriasis, and found that PE has an inhibitory

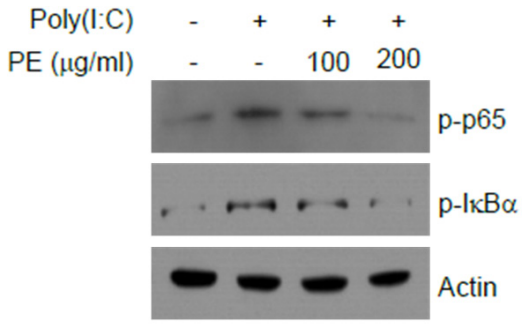

Figure 3. Effect of PE on poly (I:C)-induced NF-κB activation in keratinocytes. SV-HKEs were pretreated with PE at the indicated concentrations for 1 h, and then stimulated with 1 μg/ml poly (I:C) for 45 min. Activation of NF-κB signaling was determined by Western blot. The protein levels for phoshorylated-p65 (p-p65) and phosphorylated-IκBα (p-IκBα) were decreased by PE treatment. Actin was used for internal control.

potential on inflammatory reaction of keratinocytes. We first determined the cytotoxicity of PE on keratinocytes cultured in vitro. PE did not induce cell death up to the dose of 200 μg/ml, while it looked like that slight cell death was induced at higher dose (**Figure 1**).

Poly (I:C) is a synthetic analogue of double-stranded RNA that induces innate immune response in a TLR3-dependent manner [16]. It has been suggested that psoriatic keratinocytes show increased sensitivity to viral RNA intermediates, thereby leading to excessive proinflammatory response and maintenance of the inflammatory skin phenotype [10]. We examined whether PE affects poly (I:C)-induced immune response in keratinocytes. Poly (I:C) increased expression of inflammation-related cytokines including IL-6, IL-8, CCL20 and TNF-α from keratinocytes, while PE pretreatment significantly inhibited the poly (I:C)-induced cytokine expression (**Figure 2A**). Consistent with these results, poly (I:C) increased secretion of IL-8 and TNF-α from keratinocytes, and PE pretreatment significantly inhibited poly (I:C)-induced cytokine release (**Figure 2B**).

To investigate putative action mechanism, we examined the effect of PE on NF-κB signaling, the central player in inflammatory reaction. Poly (I:C) increased NF-κB activity in terms of phosphorylation of p65 subunit. PE pretreatment significantly inhibited poly (I:C)-induced phosphorylation of p65 in a dose-dependent manner. Consistent with this result, phosphorylation of IκBα was also increased by poly (I:C), which was markedly inhibited by PE pretreatment (**Figure 3**).

To delineate the potential action mode, we examined the effect the PE on inflammasome activation. It has been established that activation of TLR3 induces IL-1β production via an inflammasome-dependent mechanism in keratinocytes [16]. Poly (I:C) induced IL-1β secretion from keratinocytes, together with inflammasome-activated caspase-1. Pretreatment with PE significantly inhibited the secretion of IL-1β and caspase-1 (**Figure 4A**), suggesting that PE has an inhibitory potential on inflammasome activation. To further evaluate the effect on inflammasome, we determined the protein levels for NLRP3 and ASC, two important components of the innate cytosolic molecular complex of inflammasome [17]. Poly (I:C) increased NLRP3 level, which was markedly blocked by PE pretreatment. However, ASC level was not affected by both poly (I:C) and PE (**Figure 4B**). These results potentiate the fact that PE shows inhibitory effect on poly (I:C)-induced inflammatory reaction via modulation of inflammasome activity in keratinocytes.

Discussion

Among the skin-comprising cells, keratinocytes play a pivotal role for protection against various external stimuli. Keratinocytes provide protective function by both physical and functional aspects. For a physical protection, keratinocytes are committed to differentiation program and make barrier structure. For a functional defense, keratinocytes exert their role as the primary defense cells against non-self and/or self antigens. It has been established that keratinocytes express many TLR molecules and engage in innate immune response [5]. In condition related to the skin diseases, keratinocytes are susceptible to external stimuli and show excessive response. Well-known example includes psoriasis, in which keratinocytes produce a range of inflammatory cytokines, thereby functioning to recruit and activate immune cells such as neutrophils and activated T cells [18]. Thus, drug development targeting inflammatory reaction of keratinocytes is one attractable method for treatment of psoriasis. In this study, we demonstrated that PE inhibit-

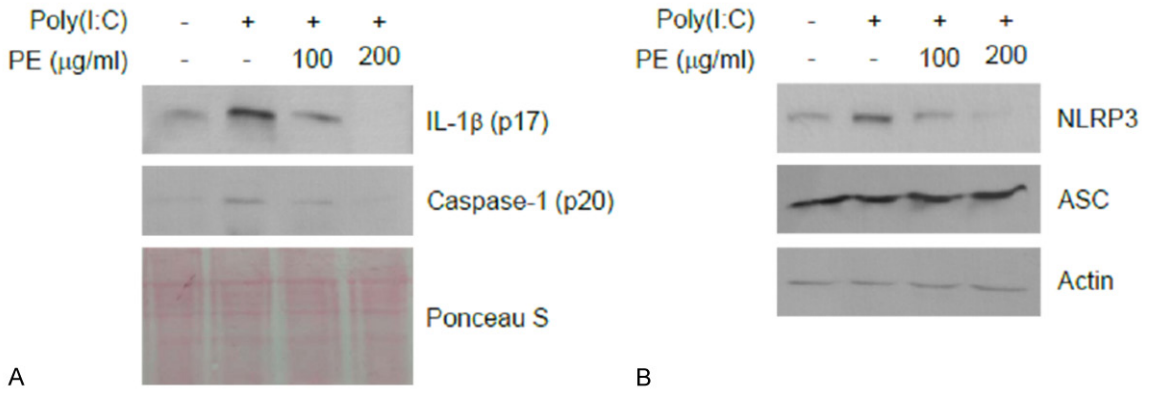

Figure 4. Effect of PE on poly (I:C)-induced inflammasome activation in keratinocytes. A. SV-HKEs were pretreated with PE at the indicated concentrations for 1 h, and then stimulated with 1 μg/ml poly (I:C) for 24 h. Culture medium was collected and concentrated, then subjected to Western blot. The secreted protein levels for IL-1β and caspase-1 were decreased by PE treatment. Ponceau S staining was used for loading control. B. The protein levels for NLRP3 and ASC, the components of inflammasome, were determined by Western blot. PE pretreatment inhibited poly (I:C)-induced NLRP3. Actin was used for internal control.

ed poly (I:C)-induced inflammatory reaction in cultured keratinocytes, suggesting that PE can be applicable for the treatment of psoriasis.

Paeonia lactiflora Pallas is a medicinal herb that has been widely used in oriental medicine. Its constituents include paeonoside, paeoniflorin, paeonol, β-sitosterol, and gallotanin. Multiple pharmacological activities, including analgesic, anti-inflammatory, and anti-metastasis effect, have been reported. For example, paeonol suppresses chondrosarcoma metastasis through up-regulation of microRNA-141 by modulating protein PKCδ and c-Src signaling pathway [19]. Other evidence shows that paeoniflorin protects against lipopolysaccharide-induced acute lung injury in mice by alleviating inflammatory cell infiltration and microvascular permeability [20]. Additionally, paeonol inhibits RANKL-induced osteoclastogenesis by inhibiting NF-κB pathway [21].

In this study, we demonstrated that PE inhibited poly (I:C)-induced activation of NF-κB. The intracellular signaling molecule NF-κB has long been recognized as the key player in the inflammatory reaction. Thus, it can simply be speculated that anti-inflammatory effect of PE may be due to its action on intracellular signaling cascade. In addition, we demonstrated that PE significantly blocked poly (I:C)-induced inflammasome activation, in terms of decreasing the secretion of IL-1β and caspase-1. Furthermore, PE decreased protein level for NLRP3, an important component of inflammasome. It has been well described that NLRP3 controls the inflammasome by regulating caspase-1 activity and IL-1β processing, and that IL-1β secreted from keratinocytes contributes to the pathogenesis of psoriasis [22]. Thus, blocking of inflammasome activation in epidermal keratinocytes can be a good strategy for the development of therapeutics on psoriasis. In this regard, potential beneficial effect of PE should be emphasized.

In summary, we demonstrated that PE has a potential for inhibiting poly (I:C)-induced innate immune response of keratinocytes. Our data suggest that PE would be a promising candidate for the treatment of psoriasis.

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Disclosure of conflict of interest

None.

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