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Characterization of retinoic acid-inducible gene-I (RIG-I) expression corresponding to viral infection and UVB in human keratinocytes

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SUMMARY

Background: Retinoic acid-inducible gene-I (RIG-I) is a cytoplasmic protein that recognizes viral double-stranded RNA to induce the type I interferon (IFN) response. In human keratinocytes, RIG-I is induced by IFN-γ and tumor necrosis factor-α stimulation, and is abundantly expressed in psoriatic keratinocytes of the spinous and basal layers.
Objective: This study investigated the effects of extraneous stimuli including viral infection and UVB exposure on RIG-I expression in human keratinocytes.
Methods: Human skin keratinocytes (HaCaT cells) were stimulated by polyinosinic–polycytidylic acid (poly(I:C)), which mimics viral infection, and UVB exposure. We assessed the expression of RIG-I and IFN-regulatory factor (IRF)-1 in HaCaT cells by RT-PCR and Western blot analysis. Moreover, we investigated the effect of IRF-1 binding site of RIG-I gene promoter on the regulation of RIG-I expression by luciferase promoter assay and electrophoretic mobility shift assay.
Results: Poly(I:C) induced RIG-I expression, while UVB inhibited basal RIG-I expression and the poly(I:C)-induced RIG-I overexpression in HaCaT cells. IRF-1, which binds to a regulatory element located on the RIG-I gene promoter, was required for both inductions of RIG-I expression. IRF-1 expression was enhanced three hours after the poly(I:C) stimulation, consistent with the RIG-I response to poly(I:C), and thereafter was suppressed. Moreover, UVB exposure promptly decreased IRF-1 expression, resulting in decreased IRF-1 protein binding to the RIG-I promoter, and consequently, decreased RIG-I expression.
Conclusion: Thus, suppression of RIG-I and IRF-1 expression caused by UVB exposure may partly explain the inhibition of skin-based immune responses, leading to viral infection and recrudescence.

1. Introduction

Survival in an environment containing a plethora of microorganisms depends on an organism's ability to establish and maintain protective mechanisms. As the outermost layer of the human body, skin provides the first major barrier against microbial attack and plays a critical role in the body's immune responses to environmental stimuli. Keratinocytes are the major cell type in skin epidermis; they are also immune-component cells due to their many immunological functions including production of cytokines and chemokines.

Viral infection is a common trigger for skin damage, and causes various skin diseases. Viral components including viral DNA and RNA are recognized by membrane and cytoplasmic receptors in cells [1]. Toll-like receptors (TLRs) are membrane-associated receptors on cell surfaces that bind pathogens and set off a signaling cascade to produce antiviral factors including cytokines and to induce inflammatory and adaptive immune responses [2,3]. TLR3 is one of several TLRs expressed in human keratinocytes; it recognizes viral double-stranded RNA (dsRNA) released by infected cells, and triggers signaling pathways leading to interferon regulatory factor (IRF)-3 and NF-κB activation as part of the innate immune response [4]. In contrast with TLRs, retinoic acid-inducible gene-I (RIG-I) recognizes viral dsRNA in the cytoplasm, stimulating cytokine production [5]. RIG-I is a member of the DExH/D-box family proteins and is designated as an RNA helicase. It plays various roles in gene expression and cellular functions in response to many RNA viruses [6,7]. RIG-I contains an N-terminal

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caspase recruitment domain and a C-terminal helicase domain; the N-terminal domain activates downstream signaling pathways, while the C-terminal region is important for recognizing dsRNA [7]. We were the first to show that RIG-I expression in human keratinocytes is significantly enhanced by interferon (IFN)-γ and tumor necrosis factor (TNF)-α stimulation [8]. Furthermore, we detected RIG-I immunohistochemically in psoriatic keratinocytes of the spinous and basal layers, suggesting that RIG-I might function not only as a RNA helicase but also as a mediator of the cytokine network in inflammatory skin diseases [8].

UVB irradiation is another major external factor contributing to epidermal damage, including skin aging and cancer, and has been known to suppress immune responses in the skin [9–11]. A representative event is the recrudescence associated with herpes simplex labialis infection. Herpes simplex virus type 1 (HSV-1) typically infects the orofacial region causing vesicular epidermal lesions and then establishing life-long latency in the nervous system. Viral reactivation can occur after particular stimuli such as exposure to UV radiation, suggesting that UV exposure suppresses the local immunity. Most likely temporary, this inhibition is sufficient to allow viral replication in the epidermis, although the exact molecular events occurring in the lesions remain unclear. We hypothesized that UVB modifies RIG-I expression in keratinocytes, leading to local immune suppression and viral reactivation. This study therefore examined the effects of viral infection and UVB irradiation on RIG-I expression in human keratinocytes.

2. Materials and methods

2.1. Cell culture and stimuli

HaCaT cells are a spontaneously immortalized, nontumorigenic human skin keratinocyte cell line. For this study, HaCaT cells were maintained in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mg/ml sodium hydrogen carbonate, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Once 60% confluent, the HaCaT cells were washed twice with sterile phosphate-buffered saline (PBS) and maintained in the growth medium for 12 h prior to addition of polyinosinic–polycytidylic acid (poly(I:C)) at various final concentrations (Sigma–Aldrich, St. Louis, USA).

Cells in PBS were irradiated with UVB using two FL20S-E lamps (Toshiba, Tokyo, Japan) that emitted wavelengths of 280–320 nm with an emission peak at 312.5 nm and had an intensity of 500 μW/cm^2 of the target area. The irradiation dose was 30 mJ/cm^2 (25 cm distance for 1 min). After UVB irradiation, the cells were cultured in the fresh growth medium. The irradiance of the UVB rays was determined with an UVR-3036/S2 radiometer and a UVB detector (Clinical Supply, Kakamigahara, Japan). For experiments involving costimulation by UVB and poly(I:C), irradiated HaCaT cells were cultured as described in growth medium containing poly(I:C) or vehicle.

2.2. RT-PCR analysis

Total RNA was isolated from cultured cells using the RNAeasy total RNA isolation kit, as recommended by the manufacturer (Qiagen, Hilden, Germany). Extracted RNA was subjected to reverse transcription using the RNA PCR Kit (AMV) ver. 3.0 (Takara, Kyoto, Japan), according to the instructions supplied by the manufacturer. Primers for RIG-I and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: RIG-I-F (5′-GCATATTGACTGGACGTGGCA-3′), RIG-I-R (5′-CAGTCATGGCTG-CAGTTCTGTC-3′), GAPDH-F (5′-CCACCCATGGCAAATTCCATGGCA-3′), and GAPDH-R (5′-TCTAGACGGCAGGTCAGGTCCACC-3′). The primers for RIG-I and GAPDH were designed to generate fragments of 644-bp and 598-bp, respectively. The amplification conditions were 94 °C for 1 min, followed by 25 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and finally, 1 cycle of 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

2.3. Western blot analysis

Whole cell lysates were made from HaCaT cells using RIPA lysis buffer (1% Nonidet P-40, 0.1% sodium deoxycholate [SDS] in PBS) with 0.57 mM phenyl methyl sulfonyl fluoride (Sigma–Aldrich), 1 mM sodium orthovanadate (Sigma–Aldrich), and protease inhibitors (Roche Diagnostics, Mannheim, Germany). The concentrations of the extracted protein were quantified by the Bradford method. Equal protein loadings (15 μg) were subjected to 8% SDS-polyacrylamide gel electrophoresis, and electroblotted to Hybond nitrocellulose membrane (Amersham Biosciences, Chandler, USA). The membranes were incubated in blocking solution (2% non-fat skim milk in 20 mM Tris [pH 7.6], 137 mM NaCl, and 0.1% Tween 20 [TBS-T]) for 1 h at room temperature, followed by an overnight incubation at 4 °C with rabbit anti-RIG-I antiserum [12] or with rabbit anti-IRF-1 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), diluted 1:10,000 or 1:500, respectively, in blocking solution. The membranes were washed four times in TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000, Amersham Biosciences) in TBS-T for 1 h at room temperature, and the bands were visualized using an ECL-Western blotting detection system (Amersham Biosciences). The membranes were reprobed with an antibody against β-actin (Sigma–Aldrich) to verify equal protein loading. Western blots were quantitated by densitometry using Scion Image 4.0.2 analysis software.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were separated from HaCaT cells with NE-PER reagent (Pierce, Rockford, USA) according to the manufacturer's instructions. The probe sequences were as follows: IRF-1E, 5′-GTTGCACTTTCGATTTTCCCTT-3′, mutant IRF-1E 5′-GTTGCACTAAC-GACTTTCCCTT-3′ (mutated nucleotides are underlined in bold) [13], and consensus IRF-1E, 5′-GGAAGCGAAAATGAAATTGAC-3′. The double-stranded oligonucleotides were labeled with biotin. The labeled probes were then incubated with nuclear extracts at room temperature for 30 min. The reaction mixture consisted of biotin 3′-labeled deoxyoligoncleotides, and 1 μg of nuclear protein extracts with 10 mM HEPES pH 7.9, 40 mM KCl, 0.4 mM DTT, 4% glycerol, and 0.4 mM EDTA. After incubation, the reaction was electrophoresed on a 5% nondenaturing polyacrylamide gel with 0.5 × TBE (100 V for 45 min). The gel contents were transferred to a nylon membrane (Pierce), and biotin-labeled DNA was detected with the LightShift chemiluminescent mobility shift assay kit (Pierce). Nuclear extracts were also incubated with a 25-fold molar excess of wild-type IRF-1E competitor, 25 to 100-fold molar excess of mutant IRF-1E competitor and consensus IRF-1E competitor.

2.5. Promoter plasmid constructs

A 1298-bp DNA fragment spanning from −1141 to +153 (+1; transcriptional initiation site) of the promoter region of the human RIG-I gene was amplified from the normal human genome using a sense primer (RIG-F: 5′-ATTCTCGAGTCTTCACAGTGAAAAA-CAAATT-3′) containing a HindIII restriction site and an antisense primer (RIG-R: 5′-AAAGCCAAGCTTCCTCTGCTTGCAGCTAGC-TACGT-3′) containing an XhoI restriction site. The cycling parameters were 94 °C for 4 min, 30 cycles of 94 °C for 1 min,