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Research paper

Increased βTrCP are associated with imiquimod-induced psoriasis-like skin inflammation in mice via NF-κB signaling pathway

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ABSTRACT

Psoriasis is a common inflammatory skin disease characterized by T cell-mediated hyperproliferation of keratinocytes, increased angiogenesis and inflammation. Accumulating evidence suggests that some keratinocyte differentiation events are controlled by the ubiquitin/proteasome system. β-transducin repeat-containing protein (βTrCP) serve as substrate recognition component of E3 ubiquitin ligases that control stability of important regulators of signal transduction including the nuclear factor (NF)-κB signaling, a key regulatory element in inflammatory pathways related to psoriasis, suggesting a potential role of βTrCP in psoriasis pathogenesis. However, no published study has investigated the role of βTrCP in the etiology of psoriasis. Here, we combined an in vitro cell model of tumor necrosis factor (TNF)-α-induced keratinocyte inflammation and an animal model of imiquimod (IMQ)-induced psoriasis-like inflammation to investigate the pathogenic mechanisms in psoriasis-like dermatitis and assess its βTrCP/NF-κB dependency. Daily application of IMQ on mouse back skin induced inflamed scaly skin lesions resembling plaque type psoriasis. These lesions were associated with elevated βTrCP levels, reduced inhibitor κB (IκB), and enhanced NF-κB activation in epidermal tissues. Furthermore, βTrCP knockdown via siRNA in in TNF-α-stimulated HaCaT and normal human epidermal keratinocytes (NHEK) cells significantly inhibited the over-activation of NF-κB and expression of intercellular adhesion molecule 1 (ICAM-1), demonstrating a pivotal role of βTrCP in regulation the TNF-α-activated NF-κB inflammatory pathways. Moreover, downregulation of βTrCP through lentiviral shRNA ameliorates IMQ-induced psoriasis-like skin lesions in vivo. In conclusion, βTrCP is involved in the NF-κB signaling mediated-, psoriasis-related inflammation and represent a novel target for developing agents to treat psoriasis.

1. Introduction

Psoriasis is a common cutaneous disorder characterized by inflammation and abnormal epidermal proliferation with a prevalence of up to 4% in the general population (Griffiths and Barker, 2007). The pathogenesis of psoriasis is multifactorial and, as a chronic inflammatory skin disorder, the imbalance between pro- and anti-inflammatory mediators may play an important role in the development and progression of this disease. Specifically, tissue alterations seen in psoriasis are driven by the exaggerated production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, and IL-12 (Bos and De Rie, 1999; Reich et al., 1999). Anti-inflammatory factors such as IL-10 are relatively decreased (Asadullah et al., 1998; Reich et al., 2002).

The nuclear factor (NF)-κB signaling activation in psoriasis development and progression has been studied intensely (for review see (Tsuruta, 2009; Goldminz et al., 2013)). NF-κB is one of the most important regulators of pro-inflammatory gene expression by synthesizing cytokines such as TNF-α, IL-1β, IL-6, and IL-8. Mammals carry 5 members of the NF-κB family including p50/p105, p65/RelA, c-Rel, RelB, and p52/p100. Although NF-κB exists in the cytoplasm as a homodimer or heterodimer composed of variable subunits: p50, p52, p65(RelA), c-Rel, and RelB, the major form of NF-κB is exists as a heterodimer between p50 and p65, encoded by the *NFKB1* and *RelA* genes, respectively (Baeuerle and Henkel, 1994; Baldwin, 1996; Lawrence et al., 2001). Currently, the immunopathogenesis theory which believes the cytokines related with highly expressed activated type 1 T (Th1) have been implicated in the processes of psoriasis, has dominated thinking on psoriasis mechanisms (Lee and Cooper, 2006; Perez-Lorenzo et al., 2006). Specifically, NF-κB acts as a central mediator which links the activated Th1 cells with the transcription of multiple genes encoded cytokines, such as TNF-α, IL-8, and IL-12, that are required for the pathogenesis of psoriasis (Victor et al., 2003; Johansen et al., 2005; Shaker et al., 2006). Importantly, a previous study has demonstrated a positive correlation between the increased expression of NF-κB and synovial membranes and lesions in the

Abbreviations: βTrCP, β-transducin repeat-containing protein; TNF, tumor necrosis factor; IMQ, animal model of imiquimod; IPI, induced psoriasis-like inflammation; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; NF, nuclear factor; Th1, type 1 T.

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psoriatic patients (Danning et al., 2000). Therefore, restoring the dysfunction of NF-κB signaling may predict a clinical benefit to the treatment of psoriasis. Indeed, recent evidence has shown that many drugs such as glycyrrhizin ameliorate psoriasis-like skin lesions by inhibiting the activation of NF-κB signaling and following expression of the adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) (Xiong et al., 2015).

β-transducin repeat-containing proteins (β-TrCP), encoded by the *β-TrCP* gene, are found to be a positive regulator of NF-κB signaling (Ougolkov et al., 2004). In the ubiquitin-proteasome pathway, β-TrCP is a substrate recognition subunit of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase (Cardozo and Pagano, 2004; Fuchs et al., 2004). By regulating substrate degradation, β-TrCP is involved in multiple cellular processes, including cell division and various signal transduction pathways including NF-κB signaling by targeting β-catenin and inhibitor κB (IκB) for proteasomal degradation. It is well known that the ubiquitin–proteasome pathway is important in regulating protein signaling pathways that are involved in some keratinocyte differentiation events (Hsieh and Lin, 1999; Jung et al., 2013). However, to our best knowledge, whether the β-TrCP is involved in the psoriasis is still unclear. And how β-TrCP/NF-κB signaling regulates pathogenesis of psoriasis-like skin lesion has not yet to be investigated.

The observations of the aforementioned studies prompted the present study, aimed at evaluating the putative role of β-TrCP in regulating NF-κB signaling using cell model of TNF-α-induced keratinocyte inflammation and an animal model of imiquimod (IMQ)-induced psoriasis-like inflammation. We found that increased βTrCP are correlated with psoriasis-like inflammation in mice via promoting NF-κB signaling, and downregulation of βTrCP ameliorates IMQ-induced psoriasis-like inflammation.

2. Materials and methods

2.1. Animal model

All animal experiments were performed in accordance with the National Institutes of Health Guidelines on Laboratory Research and approved by the Animal Care Committee at Xi'an Jiaotong University (No. 0301A0022014). All efforts were made to reduce the number of animals used and their suffering. Male BALB/c mice (8–11 weeks old) were obtained from the Animal Experiment Center at Xi'an Jiaotong University. All of the mice used for this study were kept under specific pathogen-free conditions and provided with food and water ad libitum. The mice were received a daily topical dose of 62.5 mg of 5% IMQ cream (Aldara; 3M Pharmaceuticals, UK) on a ~3 cm × 4 cm shaved back area for 7 consecutive days. Control mice were treated similarly with a control vehicle cream (Vaseline Lanette cream; Fagron). To score the severity of inflammation of the back skin, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index (PASI), which consists of measures for skin erythema, scaling and thickness. Each parameter was scored independently on a scale from 0 to 4, where 0 = no clinical signs; 1 = slight clinical signs; 2 = moderate clinical signs; 3 = marked clinical signs; and 4 = very marked clinical signs. The cumulative score denotes the severity of inflammation. At the end of the experiment on day 7, all of the mice were sacrificed, and samples of their skin were collected within 2 h for additional experiments.

2.2. Cell culture

The immortalized human keratinocyte cell line (HaCaT) was stored in our lab. HaCaT cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin. For the experiments, 1×10^5 cells/mL were seeded in a culture dish and maintained in the tissue culture incubator. TNF-α (10 ng/mL, Peprotech, USA) for 20–30 min was used for stimulation.

Neonatal human epidermal keratinocytes (NHEK) (Cascade Biologics, USA) were cultured in EpiLife medium supplemented with EDGS, 0.06 mM $CaCl_2$ (Cascade Biologics, USA) and Pen-Strep(100 units/mL Penicillin and 100 μg/mL Streptomycin). NHEK were seeded at a density of 3500 cells/cm^2 and given fresh media the day after seeding and then every 48 h until reaching 70% confluency. For the experiments, TNF-α (10 ng/mL, Peprotech, USA) was used to stimulate NHEK.

2.3. siRNA transfection

Human β-TrCP siRNA (sense, 5′-AAG UGG AAU UUG UGG AAC AUC-3′) was transfected with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For the transfections, HaCaT cells were grown in DMEM medium containing 10% FBS and plated in 6-well plates to yield a 40–60% confluence at the time of transfection. The next day, the culture medium was replaced with 500 μL of Opti-MEM (Invitrogen), and the cells were transfected using the RNAiMAX transfection reagent (Invitrogen). For each transfection, 5 μL RNAi- MAX was mixed with 250 μL of Opti-MEM and incubated for 5 min at room temperature. In a separate tube, siRNA (100 pM for a final concentration of 100 nM in 1 mL of Opti-MEM) was added to 250 μL of Opti-MEM, and the siRNA solution was added to the diluted RNAiMAX reagent. The resulting siRNA/RNAiMAX mixture (500 μL) was incubated for an additional 25 min at room temperature to allow complex formation. Subsequently, the solution was added to the cells in the 6-well plates, giving a final transfection volume of 1 mL. After incubation for 6 h, the transfection medium was replaced with 2 mL of standard growth medium, and the cells were cultured at 37 °C. The cells were replaced with growth medium after transfection for 24 h. After TNF-α treatment, the cells were then subjected to Western blot and ELISA analysis.

2.4. Lentiviral construction and infection

The oligonucleotides carrying shRNA (ATCAAGATCTGGGATAAAA) targeting both murine β-TRCP1 and β-TRCP2 were ligated into the enhanced fluorescent protein (EGFP)-tagged lentiviral vector plenti6/V5-topo. Recombinant lentiviruses were produced using the ViralPower Lentiviral Expression System (Invitrogen, K4970-00). The virus was concentrated 10 times by centrifugation in a Centricon plus-20 filter (Millipore) according to the manufacturer's instructions. Aliquots were stored at −80 °C. All virus preparations were titred according to the Virapower protocol and contained 4×10^8 TU/mL. For the in vivo treatment, mice were received intradermal back injections of lentiviral particles encodingβ-TrCP shRNA (150 μL) or control shRNA (150 μL) on their back skin. IMQ treatments were performed as previously described 1 weeks after intradermal back injections of lentiviral shRNA, Skin inflammation was scored as described before. Skin biopsies around the injection sites were collected as mentioned before.

2.5. Quantitative RT-PCR analyses

Total RNA was extracted with a RNAiso Plus kit (Takara, Shiga, Japan). cDNA was synthesized by using an oligo$(dT)_{15}$ primer and Superscript II (Invitrogen, Carlsbad, CA). Quantitative real-time reverse transcription-PCR (RT-PCR) reactions (25 μL) contained 2 μL of cDNA, 12.5 μL of SYBR Green (Applied Biosystems, Foster City, CA), and the appropriate primers. Product accumulation was monitored by SYBR Green fluorescence with ABI Prism 7000 Sequence Detection Systems. The relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. β-actin quantification was used as an internal control for normalization. Forward primers, reverse primers and probes of β-TrCP, NF-κB (p65), ICAM-1 and β-actin for real-time PCR are as follows: for β-TrCP, (forward, 5′-TGTGG CCAAA ACAAA ACTTG CC-3′; reverse, 5′-ATCTG ACTCT GACCA CTGCT C-3′); for NF-κB (p65) (forward, 5′-ATCAA TGGCT ACACA GGA-3′; reverse, 5′-CCCGT GAAAT ACACC TCA-3′); for ICAM-1 (forward, 5′-GGCTG GAGCT GTTTG