European Journal of Pharmacology 813 (2017) 33–41

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and inflammation

Imiquimod-induced psoriasis-like inflammation in differentiated Human keratinocytes: Its evaluation using curcumin

Sandeep R. Varma*,[1], Thiyagarajan O. Sivaprakasam[1], Abheepsa Mishra, Sunil Prabhu, Rafiq M, Rangesh P

Research and Development, The Himalaya Drug Company, Bangalore 562162, India

ARTICLE INFO

Keywords:
Curcumin
IL-17
Imiquimod
Inflammation
In vitro model
HaCaT
Psoriasis

ABSTRACT

Psoriasis is considered to be a systemic disease of immune dysfunction. It is still unclear what triggers the inflammatory cascade associated with psoriasis but recent evidences suggest the vital role of IL-23/IL-17A cytokine axis in etiology of psoriasis. Several studies have been conducted in psoriatic-like animal models but ethical issues and complexity surrounding it halts the screening of new anti-psoriatic drug candidates. Hence, in this study, we developed a new *in-vitro* model for psoriasis using imiquimod (IMQ) induced differentiated HaCaT cells which could be used for screening of new anti-psoriatic drug candidates. The differentiated HaCaT cells were treated with IMQ (100 μM) to induce psoriatic like inflammation and its effect was investigated using a natural anti-psoriatic compound, curcumin. The proliferation of psoriatic-like cells was inhibited by curcumin at 25 and 50 μM concentrations. The psoriatic-like cells decreased in number with increase in apoptotic and dead cells upon curcumin treatment. Curcumin inhibited the proliferation of IMQ-induced differentiated HaCaT cells (Psoriatic-like cells) by down-regulation of pro-inflammatory cytokines, interleukin-17, tumor necrosis factor-α, interferon-γ, and interleukin-6. Apart from this, curcumin significantly enhanced the skin-barrier function by up-regulation of involucrin (iNV) and filaggrin (FLG), the regulators of epidermal skin barrier. The IMQ-induced differentiated HaCaT *in vitro* model recapitulated some aspects of the psoriasis pathogenesis similar to murine model. Henceforth, we conclude that this model may be used for rapid screening of anti-psoriatic drug candidates and warrant further mechanistic studies.

1. Introduction

Psoriasis is a serious chronic inflammatory skin condition affecting 2–3% of the total populations worldwide *i.e.* 125 million people all over the world suffer from psoriasis, according to World Psoriasis Day consortium. (Stern et al., 2004; Wagner et al., 2010). The occurrence of the disease is believed to be multifactorial involving genetic and environmental factors. It is usually distinguished by hyperproliferative keratinocytes and invasive leukocytes. Earlier, the three phase model of psoriasis pathogenesis that includes sensitization phase, silent phase, and effectors phase has been described (Sabat et al., 2007). The exact etiology behind psoriasis is not well-understood but recent clinical evidence suggests the crucial role of activated T-cells and cytokines in the inflammatory cascade. However, upcoming research evidence highlights the crucial role of IL-23/IL-17A cytokine in progression of the disease (Di Cesare et al., 2009; Zaba et al., 2009). Several immune-related cytokines such as IL-23, IL-17A, IL-20, IL-22, IL-1β, IL-6, and TNF-α play a vital role and share intricate relationship in pathogenesis of psoriasis (Kuhn and Luger, 2010; Sun et al., 2013). The growing evidence suggests that the dysregulation of IL-17A signaling and uncontrolled activation of Th17 pathway promotes the pathogenesis of several autoimmune and inflammatory disorders including psoriasis (Elloso et al., 2012). Apart from this, during psoriasis, impairment of skin barrier occurs that alters the structure and function of the skin. Thus, the structural and functional alteration of skin leads to decrease in skin-barrier function proteins namely, involucrin and filaggrin.

Curcumin (*Curcuma longa* L.), one of the principal constituent of turmeric displays plethora of biological activities like anti-inflammatory, antioxidant, anticancer, antiviral, antimicrobial, and hepatoprotective properties (Akram et al., 2010). The non-toxic nature of curcumin makes it a potential target for the treatment of various skin disorders like psoriasis, scleroderma, and skin cancer. The inhibitory effects of curcumin on inflammatory cascade are delivered through multiple mechanisms such as suppression of NF-κB, down-regulation of

* Correspondence to: Department of Cell Biology, Research and Development, The Himalaya Drug Company, Makali, Bangalore 562162, India.
E-mail addresses: dr.sandeepvarma@himalayawellness.com, sandeepravivarma@gmail.com (S.R. Varma).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.ejphar.2017.07.040
Received 18 May 2017; Received in revised form 20 July 2017; Accepted 20 July 2017
Available online 21 July 2017

IL-1β, IL-6, and TNF-α, and inactivation of STAT-3 pathway that might lead to suppression of psoriatic inflammation and hyper proliferation. The recapitulation of psoriatic-like inflammatory condition has been made possible by using an immune-regulator Imiquimod (IMQ) in mice model. IMQ, TLR7/ TLR8 ligand is used for topical treatment of virus-related skin diseases, actinic keratosis, and basal cell carcinomas (Beutner and Tyring, 1997; Geisse et al., 2002; Szeimies et al., 2004). Recently, topical administration of 1% curcumin gel in IMQ-induced psoriatic mice model led to suppression of inflammatory cytokines IL-1β and IL-6. The ethical issues and complexity associated with the use of animal models hinders in rapid screening of potential anti-psoriatic drug candidates.

The aim of the present study was thus to develop a new *in vitro* model for screening anti-psoriasis drug candidates using IMQ induced differentiated human keratinocytes. The study investigated the modulatory role of curcumin in inhibiting cell proliferation, inflammation, and enhancing the skin barrier function using this model.

2. Methods

2.1. Chemicals and reagents

Ham's F12, Stemline Keratinocyte Medium II, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (PubChem CID: 16218671), Dimethyl sulfoxide (DMSO) (PubChem CID: 679), Neutral Red (PubChem CID: 11105), Trichloroacetic acid (PubChem CID: 6421), Acetic acid (PubChem CID: 176), Tris base, Calcium chloride (PubChem CID: 5284359), Imiquimod (IMQ) (PubChem CID: 57469), and Curcumin (PubChem CID: 969516) were purchased from Sigma Aldrich, Bangalore, India. Fetal Bovine Serum (FBS) was purchased from Invitrogen, USA. Chelex-100 resin was purchased from Bio-Rad, USA. ELISA kits for human IL-17, TNF-α, IFN-γ, IL-6, were purchased from Invitrogen, USA. ELISA kits for human Involucrin and Filaggrin were purchased from Cloud-Clone Corp., USA. Live-Dead cell assay kit was purchased from PromoKine, Germany. All the molecular biology reagents for qPCR were obtained from Bio Rad, USA.

2.2. Cell culture and its maintenance

Human keratinocytes (HaCaT) were obtained from National Centre for Cell Science, Pune, India., and grown in Ham's F12 medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C with 5% CO_2. In the experiment, 2% serum with Ham's F12 medium was used maintenance medium, as it was necessary to synchronize the cells in same cell cycle phase prior to treatment.

2.3. Molecular docking studies

Molecular docking was performed with energy minimized structures of protein and ligands. Using AutoDock Vina, the energy-minimized structure of curcumin was docked with IL-17, TNF-α, IFN-γ, IL-6, FLG, and iNV (Trott and Olson, 2010). The protein and ligand files were converted into a PDBQT file format, having atomic charges and definitions for ligands including structural information. After receptor–ligand preparation and specifying the binding site, program for docking was run from the command prompt (Mishra et al., 2014). The binding energy was calculated for the docked complex (Receptor-ligand) and represented as affinity *(kcal/mol)*.

2.4. Cell viability assay

The viability of cells was determined by MTT assay to reveal the initial cell death (Mosmann, 1983). HaCaT cells were cultured in 96-well plates (1×10^4 cells/ml) and treated with various concentrations of curcumin ranging from 15.625 to 1000 µg/ml. After 24 h incubation, cytotoxicity assay was assessed by MTT (10 µl well containing 5 mg/ml of stock in PBS; 100 µl of cell suspension) solution and absorbance was monitored at 540 nm using Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT). The non-toxic concentrations of curcumin were further used in subsequent experiments.

2.5. Cell differentiation assay

To set up an efficient and consistent model for psoriasis *in vitro,* differentiated HaCaT cells was used. HaCaT cells (1×10^5 cells/ml) were cultured in 40 mm petridishes with Stemline Keratinocyte Medium II (calcium free), with 10% chelexed FBS and incubated for 24 h at 37 °C with 5% CO_2. FBS was calcium depleted with Chelex-100 resin according to the manufacturer's protocol (Bio-Rad, USA). HaCaT cells were incubated with 2 mM calcium chloride ($CaCl_2$) and further incubated for 4 h to achieve differentiation. After exposure to $CaCl_2$, the morphological changes were observed and images were captured under phase contrast microscope (Fig. S1). These differentiated HaCaT cells were taken for further experiments. IMQ (100 µM) was used to induce psoriatic like inflammation in differentiated HaCaT cells (Li et al., 2013).

2.6. Cell proliferation assay

HaCaT cells were seeded in 96-well plates (1×10^4 cells/ml) and incubated for 24 h at 37 °C with 5% CO_2. After differentiation, the cells were treated with various concentrations of curcumin (1.56–50 µg/ml) and IMQ 100 µM concentration respectively. Further, the cells were incubated for 24 h and the cell proliferation activity was determined by employing MTT (Mosmann, 1983), SRB (Vichai and Kirtikara, 2006), and NRU (Repetto et al., 2008) assay. The absorbance was read at 540 nm using Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT).

To estimate the total DNA content in the treated/untreated cells, differentiated HaCaT cells were plated in 40 mm petri plates with an initial seeding density of 1×10^5 cells per ml in serum free media and incubated with/without curcumin in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h. Total DNA was extracted from the treated/untreated cells using TRI reagent and the DNA was quantified using a spectrophotometer at 260 and 280 nm and the total yield/number of cells were calculated. The total DNA yield with different doses of curcumin was compared with the untreated cells and the fold increase/decrease was calculated.

2.7. Morphology of IMQ-induced differentiated HaCaT Cells

2.7.1. Phase contrast microscopy

Briefly, IMQ-induced differentiated HaCaT (1×10^5 cells/ml) cells were treated with or without two different concentrations of curcumin (25 µM and 50 µM) at 37 °C for 24 h. The curcumin treated HaCaT cells were washed twice with PBS and were viewed under phase contrast microscope.

2.7.2. Neutral Red Staining

Briefly, HaCaT cells (1×10^5 cells/ml) were seeded into 6-well plates for overnight incubation at 37 °C with 5% CO_2. After differentiation, the cells were treated with IMQ (100 µM) and two non-toxic concentrations of curcumin (25 µM and 50 µM) respectively. Then the cells were further incubated for 24 h at 37 °C. The cells were washed twice with 1X PBS post-incubation, fixed with 3.7% paraformaldehyde, and stained with neutral red dye for 60 min (Landua et al., 2009). The cells were again washed thrice with 1X PBS and visualized under the phase contrast microscope.