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Ameliorating effect of TI-1-162, a hydroxyindenone derivative, against TNBS-induced rat colitis is mediated through suppression of RIP/ASK-1/MAPK signaling

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ABSTRACT

The pathogenesis of inflammatory bowel disease (IBD) is associated with production of immense pro-inflammatory cytokines including TNF-α. Once generated, TNF-α stimulates production of various pro-inflammatory cytokines and disrupts mucosal barrier by inducing inflamed mucosal epithelial cell death. In the present study, we investigated inhibitory effects of TI-1-162, a hydroxyindenone derivative, against TNF-α-induced and TNBS-induced colon inflammation. TI-1-162 showed inhibitory effect on the TNF-α-induced adhesion of U937 monocytic cells to HT-29 colonic epithelial cells (IC_{50} = 0.83 ± 0.12 μM), which is an *in vitro* model representing the initial step of colitis. In addition, TI-1-162 suppressed TNF-α-stimulated caspase-3 activation and HT-29 cell apoptosis. These *in vitro* inhibitory activities of TI-1-162 correlated to recovery changes in *in vivo* colon tissues, such as downregulation of adhesion molecules (ICAM-1, VCAM-1) and chemokines (CCL11, CXCL1, CXCL2, CXCL3, CX3CL1) revealed by gene expression array and Western blot analyses. Such molecular recovery of colon epithelium from TNBS-treated rats corresponded to the recovery in body weight, colon weight/length, and myeloperoxidase level by TI-1-162 (10 and 30 mg/kg/day, orally). In relation to action mechanism, TI-1-162 did not disturb TNF-α binding to its receptor, but suppressed phosphorylation of RIP-1, ASK-1, JNK and p38, and nuclear translocation of NF-kB and AP-1, which corresponded to down regulation of inflammatory cytokines in TNF-α-treated cells (HT-29 and U937) and TNBS-treated rat colon tissues. Taken together, the results indicate that the protective effects of TI-1-162 against colon inflammation and epithelial cell death are associated with its inhibitory action in RIP/ASK-1/MAPK signaling pathway downstream to TNF receptor 1.

1. Introduction

Inflammatory Bowel Disease (IBD), encompassing Crohn's Disease (CD) and Ulcerative colitis (UC), describes chronic and progressive inflammation of the digestive tract (Yapali, 2007). Both CD and UC usually involve severe diarrhea, abdominal pain, fatigue and weight loss, which may lead to debilitating complications. The etiology of IBD has not been completely revealed, however, aberrant and prolonged immune response triggered by genetic and environmental factors are considered as the major etiology of the disease (Danese et al., 2004; Rocchi et al., 2012). IBD is often characterized by an infiltration of mucosal immune cells (macrophages, neutrophils and lymphocytes) into the intestinal tissue producing a wide range of pro-inflammatory cytokines, resulting in imbalance between pro- and anti-inflammatory cytokines (Neurath, 2014a; Rogler and Andus, 1998). The increased levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ are mostly responsible for the pathogenesis of IBD (Schreiber et al., 1995; Zhang and Li, 2014).

In the inflamed mucosa of patients with IBD, TNF-α exerts various functions such as stimulating production of other pro-inflammatory cytokines and chemokines, disrupting barrier function, and promoting cell death of intestinal epithelial cells (Neurath, 2014a; Parameswaran and Patial, 2010). Binding of TNF-α to its receptor (TNFR) induces receptor trimerization and recruits downstream adaptor molecules, such as TNF receptor type 1-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor interacting protein-1 (RIP-1). In turn, RIP-1 recruits mitogen-activated protein kinase kinase and p38 to activate inhibitor of κB kinase (IKK) complex

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which phosphorylates IκB leading to IκB degradation and consequent nuclear translocation of NF-κB (Blüml et al., 2012; Parameswaran and Patial, 2010). Germinal center kinase (GCK) coupled to TRAF2 also activates mitogen-activated protein kinase/ERK kinase kinase 1, Jun N-terminal kinase (JNK), and p38 (Sethu and Melendez, 2011; Yuasa et al., 1998). Apoptosis-signaling kinase-1 (ASK-1) is also activated either by interaction with TRAF2 and TRADD or by reactive oxygen species generated after TNF receptor 1 ligation, which ultimately leads to activation of JNK and AP-1 (Geering et al., 2011; Geering and Simon, 2011; Papa et al., 2009; Parameswaran and Patial, 2010; Westwick et al., 1994). In addition, recruitment of TRADD and Fas associated protein with death domain (FADD) by trimerized TNF receptor 1 forms death-inducing signaling complex (DISC) to activate pro-caspase-8 leading to activation of apoptosis executioner, caspase-3 (Hsu et al., 1996; Sedger and McDermott, 2014).

Reflecting the critical role of TNF-α in the pathogenesis of IBD, biological drugs such as infliximab and adalimumab targeting and blocking TNF-α action have become a mainstay in the therapy of IBD over other orally available drugs such as aminosalicylate, glucocorticoids, and immunosuppressive drugs. However, the biological drugs have a high level of economic burden and low degree of administration compliance. In addition, the biological drugs exhibit deleterious side effects (Bernstein, 2015). In the current treatment regimens for IBD, it is required to develop orally available new drugs to have better efficacy including epithelium recovery and less side effect. Previously, we have investigated 2-benzylidene-2,3-dihydro-1*H*-inden-1-one and benzofuran-3(2*H*)-one derivatives for their structure-activity relationships (SAR) against TNF-α action (Kadayat et al., 2017). According to our previous study, TI-1–162 seems to be a promising lead compound for IBD drug development. However, no data exist that shed light on its efficacy and action mechanism. In the current study, we examined protective effects of TI-1-162 against inflammation and epithelial apoptosis in *in vitro* and *in vivo* IBD models, and its mode of action.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all the materials were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). RPMI-1640, fetal bovine serum (FBS), penicillin/streptomycin and trizol reagent were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Trypsin/EDTA was purchased from Clonetics, Inc. (Walkersville, MD, USA). Antibodies directed against TNF-α, IL-1β, ICAM-1, VCAM-1, β-actin and Lamin B were purchased from Abcam (Cambridge, MA, USA) whereas antibodies of phospho-I-κB, I-κB and c-Fos were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NF-κB, ASK1, phospho-ASK1, JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, RIP-1, phospho-RIP-1, p38 MAPK, phospho-p38 MAPK, and c-Jun antibodies were sourced from Cell Signaling Technology Inc (Beverly MA, USA) while CX3CL1 and CCL11 antibodies were obtained from Biorbyt Ltd (San Francisco, CA, USA) and antibodies against IL-8 and IL-6 were purchased from Abbiotech (San Diego, CA, USA). 5-Aminosalicyclic acid (5-ASA), Lipopolysaccharide (LPS), TPA (12-O-tetradecanoylphorbol-13-acetate) and Sulfasalazine (SSZ) were received from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human TNF-α was procured from R and D system Inc, (Minneapolis, MN, USA). BCECF/AM (acetoxymethyl ester) was received from Molecular probes (Eugene, Oregon, USA).

TI-1–162 is an analog of 2-benzylidene-2,3-dihydro-1*H*-inden-1-one, which was designed and synthesized for the discovery of orally available new chemical entity for the treatment of inflammatory bowel disease. TI-1-162 for the current study was prepared by Professor Eung-Seok Lee and Dr. Tara Man Kadayat with the same method as reported previously (Kadayat et al., 2017).

2.2. Cell culture

Human colorectal cancer cell line, HT-29 and human pre-monocytic cell line, U937 were obtained from the American Type Culture Collection (Manassas, VA, USA). HT-29 cells and U937 cells were cultured in RPMI-1640 media containing 10% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. Cells were maintained at 37 °C in 5% CO_2 in humidifier incubator.

Differentiation of U937 cells was done by using 20 ng/ml of TPA (12-O-tetradecanoylphorbol-13-acetate) for 48 h. Then the adhered TPA-differentiated U937 cells were further pretreated with TI-1-162 compound for 1 h before treatment with LPS (1 μg/ml) and TNF-α (10 ng/ml).

2.3. Monocyte-epithelial cell adhesion assay

Monocyte to colon epithelial cell adhesion assay was performed by using U937 pre-monocytic human cells prelabeled with 2′, 7′-bis (2-carboxyethyl)-5(6) carboxyl fluorescein acetoxymethyl ester (BCECF/AM, 10 μg/ml) as previously reported (Thapa et al., 2009, 2008) with slight modification. HT-29 cells (2×10^5 cells/well) cultured in 48-well plates were pretreated with TI-1-162 for 1 h. The prelabeled U937 cells were centrifuged and then seeded (5×10^5 cells/well) on the monolayer of HT-29 cells and the co-culture was treated with TNF-α (10 ng/ml) for 3 h at 37 °C. Non adhering U937 cells were removed by washing thrice with PBS. Cells were photographed by using the Florescence microscope and then lysed with 0.1% Triton X-100 in Tris (0.1 M) in shaker for 30 min at room temperature. The fluorescence intensity was then measured using Fluostar Optima microplate reader (BMG LABTECH GmbH, Germany) at an excitation and emission wavelengths of 485 and 520 nm, respectively.

2.4. TNF receptor binding assay

TNF receptor binding assay was performed in accordance with the manufacturer's guideline using TNF-α biotin kit (R and D Systems, Minneapolis, MN, USA). Briefly, HT-29 cells were seeded at a cell density of 4×10^6 cells/ml and were treated with TI-1-162 for 1 h. The trypsinized cells were washed with 10 mM PBS and incubated with or without biotinylated rhTNF-α (10 μl) for 1 h at 2–8 °C. Then, 10 μl of avidin-FITC reagent was added for 30 mins at 2–8 °C in dark. The cells were then washed with 2 ml of 1 × RDF1 buffer and resuspended in same buffer for analysis by flow cytometer at 488 nm.

2.5. Western blot analysis

Total protein was extracted by using Radioimmunoprecipitation assay (RIPA) buffer (150 mM Sodium chloride, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS and 50 mM Tris adjusted to pH 8.0) containing 1 × proteases and phosphatase inhibitor (Thermo Scientific, Rockford, USA). Cytosolic and nuclear proteins were extracted by using manufacturer's instruction given by NE-PER nuclear and cytoplasmic extraction reagent kit (#78833, Thermo Scientific, Rockford, USA). BCA protein assay kit (Pierce, Rockford, USA) was used for quantitation of the protein content. Protein samples were separated by using SDS-PAGE and transferred to nitrocellulose membrane for up to 1 h. Blocking was done by using 5% Bovine Serum Albumin in 1 × Tris-buffered saline (TBS) Tween-20 (TBS-T) at room temperature for 1 h. The membranes were then incubated with primary antibodies overnight at 4 °C followed by washing the membrane three times with 1 × TBS-T, incubated with horseradish peroxidase-conjugated secondary antibody in 1 × TBS for 1 h at room temperature. The membrane was visualized using enhanced chemiluminescent reagent (Thermo Scientific, Rockford, USA) system on a luminescent image analyzer, LAS-4000 mini (Fujifilm, Tokyo, Japan).