Novel Compound SK-1009 Suppresses Interleukin-6 Expression through Modulation of Activation of Nuclear Factor-KappaB Pathway

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Although interleukin-6 (IL-6) is an important biological mediator playing an indispensable role in inflammation and cancer, few inhibitors and suppressors are known. In the present study, the underlying mechanisms of a novel chemically synthesized compound SK-1009, which has suppressive properties on IL-6 production in human macrophage cells, were examined. SK-1009 suppressed IL-6 mRNA levels in human colon cancer cells. Thus, the influence of SK-1009 on transcription factor, nuclear factor-kappaB (NF-κB), which is involved in expression of the IL-6 gene was assessed. SK-1009 was found to suppress degradation of IκB, an NF-κB inhibitory factor, and consequently inhibited the NF-κB activation pathway. The inhibitory property was almost the same as other NF-κB inhibitors, such as 5HPP-33. Thus, SK-1009 exerts a potent inhibitory effect on IL-6 expression, apparently mediated by modulation of activation of NF-κB transcription factor.

Key words SK-1009; interleukin-6; nuclear factor-kappaB; colon

Interleukin 6 (interferon beta 2, IL-6) is a cytokine primarily produced at acute and chronic inflammation sites, and acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 stimulates acute phase protein synthesis and neutrophil production in bone marrow. Moreover, it supports B cell growth and antagonizes regulatory T cells. IL-6 elicits its signals through a receptor complex of IL-6Rα and gp130. The function of the IL-6 gene is implicated in a wide variety of inflammation-associated diseases, such as diabetes mellitus, systemic juvenile rheumatoid arthritis and cancer.1–3

Patients with advanced and/or metastatic cancer have been reported to show high levels of IL-6 in their blood.4–8 In animal models, Apc-deficient Min mice lacking the IL-6 gene decreased overall polyp numbers by 32% compared with IL-6 wild Min mice. The Min mouse has a mutation in the Apc tumor suppressor gene and develops intestinal polyps, and shows a 10-fold increase in serum IL-6 levels at 26 weeks of age.9 On the other hand, pitavastatin is a novel chemically synthesized hydroxymethylglutaryl (HMG)-CoA reductase inhibitor, which was demonstrated to reduce intestinal polyp formation in Min mice. In the pitavastatin experiment, serum IL-6, leptin, and MCP-1 levels were decreased at a dose of 40 ppm.10 Thus, IL-6 has been suggested to play an important role in colon/intestinal carcinogenesis.

Hence, there is an interest in developing anti-IL-6 agents for therapy against inflammation and cancer.11,12 For instance, tocilizumab has been approved as a therapeutic agent for systemic juvenile rheumatoid arthritis. Indeed, it may be difficult to develop a small molecule, that selectively suppress IL-6 expression, but there are a very few small molecules reported to suppresses IL-6 expression. One of them is SK-1009 (Fig. 1). SK-1009 is a chemically synthesized compound, obtained from a screening of compound library. It has been reported to inhibit lipopolysaccharide (LPS)-induced IL-6 production in human macrophage cells, THP-1 (information from international patent WO2007/091313). However, underlying mechanisms of IL-6 suppression by SK-1009 are not elucidated in detail.

In the present study, we aimed to clarify the mechanisms by which SK-1009 suppresses IL-6 expression. We have investigated the effects of SK-1009 on IL-6 and tumor necrosis factorα (TNFα) expression levels in a human colon cancer cell line stimulated by TNFα. We found that SK-1009 effectively suppressed IL-6 expression through inhibition of activation of a transcription factor, nuclear factor-kappaB (NF-κB).

MATERIALS AND METHODS

Cells and Test Compounds Human colon cancer cell lines, HCT116, RKO and SW480 cells (American Type Culture Collection, Manassas, VA, U.S.A.), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, U.S.A.) and antibiotics at 37°C in a humidified incubator.

Fig. 1. Chemical Structure of SK-1009

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with 5% CO₂. The IL-6 suppressor SK-1009, N-hydroxy-4,5-
diphenyl-2-oxazolohexanamide, was chemically synthesized at Shizuoka Coffein Co., Ltd. Its structure is shown in Fig. 1. 

In vitro data showed that this compound reverse the inhibitory effects of LPS-induced IL-6 protein production in human macrophage cells, THP-1. IC₅₀ for SK-1009 is 2.7 × 10⁻⁷ M, and was reported in international patent WO2007/091313. TNFα was purchased from PEPROTECH (Rock Hill, NJ, U.S.A.), NF-κB inhibitors, 5HPP-33 and SM-7368 was purchased from Merck KGaA (Darmstadt, Germany). Catechin, dexemethasone and troglitazone were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Viability Determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay An hour before the end of treatment with TNFα and SK-1009, 0.5 mg/mL of MTT was added to the medium and incubated. The MTT formazan produced by living cells was dissolved with dimethyl sulfoxide and the absorbance at 590 nm was measured on a microplate reader.

Real-Time Polymerase Chain Reaction (PCR) Analysis Total RNA was isolated from cells using Isogen (Nippon Gene, Tokyo, Japan), treated with DNase (Invitrogen, Grand Island, NY, U.S.A.) and 3 μg aliquots in a final volume of 20 μL were used for synthesis of cDNA using an Omniscript RT Kit (Qiagen, Hilden, Germany) and primed primer. Real-time PCR was carried out using a DNA Engine Opticon TM 2 (MJ Research, Waltham, MA, U.S.A.) with SYBR Green Realtime PCR Master Mix (Toyobo Co., Osaka, Japan) according to the manufacturer’s instructions. Primers for NF-κB transcriptional activity, colon cancer cell

Luciferase Assay for NF-κB Transcriptional Activity To measure NF-κB transcriptional activity, colon cancer cell lines HCT116, RKO and SW480 were seeded in 96-well plates (2 × 10⁵ cells/well). After 24 h incubation, cells were transiently transfected with 25 ng/well of pGL4.32 [luc2P/NF-κB RE/Hygro] (Promega Co., Madison, WI, U.S.A.) reporter plasmid and pGL4.73 [hRluc/SV40] (Promega) control plasmid using FuGENE® 6 Transfection Reagent (Roche Inc., Basel, Switzerland) according to the instructions provided by the manufacturer, and cultured for 24 h. The cells were then treated with test agents, and finally Firefly luciferase and Renilla luciferase activities were determined by Luciferase Assay Systems and Renilla Luciferase Assay Systems (Promega), respectively. Basal luciferase activity of untreated cells was set as 1.0. The percent luciferase activity with each treatment was calculated from data for triplicate wells. The value was normalized by Renilla luciferase activity. All experiments were repeated at least three times with nearly identical results. Data are expressed as means ± S.D. (n=3).

Experiment for the Effects of Test Compounds on NF-κB Transcriptional Activity Plasmid-transfected HCT116, RKO and SW480 cells were cultured in the presence of 25 ng/mL TNFα for 2 or 6 h after incubation with 25, 50, 100 μM SK-1009. Similarly, 5HPP-33 (25, 50 μM), SM-7368 (25, 50 μM), catechin (100 μM), dexamethasone (10 μM) and troglitazone (10 μM) were added to the medium 30 min before TNFα stimulation. NF-κB transcriptional activity was normalized for transfection rate calculated by SV40 promoter driven Renilla luciferase.

Immunofluorescence Analysis in Cells HCT116, RKO and SW480 cells were seeded at a density of 8 × 10⁵/well in 8-chamber slides, and incubated in the presence or absence of 25 μg/mL TNFα for 30 min after 30 min pretreatment with or without 50 μM SK-1009. After incubation, cells were fixed with 2% paraformaldehyde for 30 min at room temperature and permeabilized with methanol for 10 min at −20°C. Cells were subjected to immunofluorescence analysis with anti-NF-κB (p65) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) at 1:100 dilutions followed by anti-rabbit immunoglobulin G (IgG) fluorescent isothiocyanate (FITC) conjugated antibody (Biotium, Hayward, CA, U.S.A.).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis The cells were lysed in SDS sample buffer solution (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) and the lysates were briefly sonicated and boiled for 5 min. Proteins from lysates of HCT116, RKO and SW480 cells were separated on polyacrylamide gels and electrophoretically transferred to Immobilon-P membranes (Merck Millipore, Billerica, MA, U.S.A.). After blocking with 2% non-fat skim milk in TTBS (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 0.05% Tween 20) for 1 h the membranes were incubated with primary antibody in TTBS containing 1% bovine serum albumin, washed with TTBS and incubated with the second antibody, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Bioscience, Little Chalfont, U.K.) and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY, U.S.A.). The antibodies were stripped by placing the blots in boiling water for several minutes and then the blots were re-probed with further antibodies: mouse monoclonal anti-β actin (Sigma Life Science, St. Louis, MO, U.S.A.) and rabbit polyclonal anti-1-xBa antibody (Santa Cruz Biotechnology Inc.).

Transcriptional Factor Activation Profiling Analysis Activation of several receptors, such as androgen receptor (AR), constitutive active/androstane receptor (CAR), estrogen receptor (ER), glucocorticoid receptor (GR), hepatocyte nuclear factor-4 (HNF4), peroxisome proliferator-activated receptor (PPAR) and progesterone X receptor (PXR) by SK-1009 in HCT-116 cells were evaluated using Transcriptional Factor Activation Profiling Array (Signosis, Inc., Sunnyvale, CA, U.S.A.). HCT116 cells (3 × 10⁶ cells/well) were cultured with or without 50 μM SK-1009 for 6 h, and then nuclear extracts were obtained by a Nuclear/Cytosol Fraction Kit (BioVision, Milpitas, CA, U.S.A.). Nuclear extracts were incubated with consensus sequences of nuclear receptor DNA-binding sites for determination of transcriptional activities according to the manufacturer’s protocol.

Statistical Analysis All the results are expressed as mean ± S.D. values, with statistical analysis using Bonferroni z-test. Differences were considered to be statistically significant at p<0.05.
RESULTS

Suppression of Cytokine Expression by SK-1009 in TNFα-Stimulated Human Colon Cancer Cells To confirm that SK-1009 suppresses IL-6 expression levels in colon epithelial cells, HCT116 and RKO cells were stimulated with 25 ng/mL TNFα to induce IL-6 and TNFα mRNA and simultaneously incubated with 25 μM SK-1009. As shown in Fig. 2, SK-1009 treatment suppressed IL-6 and TNFα mRNA levels in TNFα-stimulated cells.

Inhibition of TNFα-Treated NF-κB Transcriptional Activity by SK-1009 in Human Colon Cancer Cells NF-κB is the major transcriptional factor responsible for induction of IL-6 gene expression induced by LPS and inflammatory cytokines. Thus, we assumed that the one of the targets of SK-1009 is NF-κB, and examined the effects of SK-1009 on TNFα-activated NF-κB transcriptional activity using luciferase reporter gene assay. No significant difference was observed in the basal NF-κB transcriptional activity of HCT116 cells between serum-deprived cultures and 5% fetal bovine serum (FBS)-supplemented cultures after 6 h. To examine the effective dose and timing of TNFα, cells were cultured in a medium containing 5, 25 and 100 ng/mL TNFα without FBS, and NF-κB transcriptional activity were tested. Treatment of HCT116 cells with 5, 25 and 100 ng/mL TNFα for 6 h increased the activity to almost 7, 12 and 13 times of the control value, respectively (Suppl. Fig. 1A). NF-κB transcriptional activities after 6 h treatment with TNFα were elevated compared to those of 2 h TNFα treatment in RKO cells and SW480 cells (Suppl. Figs. 1B, C). Moreover, TNFα seems to activate NF-κB transcriptional activities to its maximum at the dose of 25 ng/mL. No significant decrease of cell viability, evaluated by MTT assay, was observed after 6 h culture with TNFα at these concentrations.

The effects of SK-1009 on TNFα-stimulated NF-κB transcriptional activity were next examined, and revealed that SK-1009 significantly suppressed TNFα-stimulated NF-κB transcriptional activity in a dose-dependent manner (Fig. 3). Decreases in TNFα-stimulated NF-κB transcriptional activity at the highest doses of SK-1009 were as follows: 64% for HCT116 cells, 42% for RKO cells and 59% for SW480 cells (Figs. 3A–C). No significant decrease of cell viability, evaluated by MTT assay obtained from Renilla Luciferase Assay Systems and MTT assay, was observed after 6 h culture with TNFα plus SK-1009 at these concentrations.

Inhibition of TNFα-Induced NF-κB Nuclear Translocation and Degradation of the Inhibitory Subunit I-κBa by SK-1009 Treatment Translocation of NF-κB to the cytoplasm is an essential step in NF-κB transcriptional activity. The phosphorylation of I-κBα by IκB kinase (IKK) results in its degradation, allowing NF-κB to enter the nucleus. We examined the effects of SK-1009 on TNFα-induced I-κBα degradation and nuclear translocation of NF-κB.

Fig. 2. Effects of SK-1009 on Various Cytokine mRNA Levels
HCT116 (A, C) and RKO (B, D) cells were cultured with and without 25 ng/mL TNFα for 6 h after 30 min incubation with 25 μM SK-1009 (SK). Relative IL-6 (A, B) and TNFα (C, D) mRNA expression levels are plotted as the ratio of the unstimulated control culture value. Data are means±S.D. (n=3). Similar results were obtained from more than three separate experiments.
nucleus activates transcription of NF-κB-responsive genes. Thus, we performed fluorescent immunohistochemical analysis of NF-κB subcellular localizations after TNFα treatment in HCT116 cells. TNFα caused NF-κB nuclear translocation within 30 min (Figs. 4A–C) and this was prevented by 50 µM SK-1009 pretreatment for 30 min. Similar results were obtained in SW480 cells, but failed in RKO cells (data not shown).

Translocation of NF-κB to the nucleus is preceded by phosphorylation and proteolytic degradation of the inhibitory subunit IκBα. To determine whether the effect of SK-1009 on NF-κB activation is related to IκBα degradation, the IκBα protein level was examined by Western blot analysis.

As shown in Fig. 4D, stimulation of the HCT116 cells with 10 ng/mL TNFα caused a rapid decrease in the abundance of IκBα protein, which almost completely disappeared in stimulated cells 10–30 min after IκBα stimulation. Similar results were obtained in SW480 cells (data not shown). However, IκBα degradation was weakly observed in RKO cells (Fig. 4E).

Effects of NF-κB Inhibitors on TNFα-Treated NF-κB Transcriptional Activity in HT116 Cells The effects of the available NF-κB inhibitors on TNF-stimulated NF-κB transcriptional activity were examined for the comparison of their potential to those of SK-1009. All the NF-κB inhibitors significantly suppressed TNFα-stimulated NF-κB transcriptional activity in a dose-dependent manner. Decreases in TNFα-stimulated NF-κB transcriptional activity at a dose of 50 µM were as follows; 67% for SK-1009, 65% for 5HPP-33 and 96% for SM-7368 (Fig. 5). SM-7368 showed the strongest inhibition and similar inhibitory potential were observed between SK-1009 and 5HPP-33.

Effects of SK-1009 on Activation of Nuclear Receptor Subfamily, Including Xenobiotic Receptors To examine the possibility that SK-1009 could be the ligand for other receptors that affect NF-κB transcriptional activity, we evaluated direct activation of several nuclear receptors, such as AR, CAR, ER, GR, HNF4, PPAR and PXR by SK-1009 in HCT-116 cells, and found no activation was observed in such receptors (Suppl. Fig. 2A). Furthermore, the effects of several agents that regulate xenobiotic metabolism, on TNFα-stimulated NF-κB transcriptional activity in HCT-116 cells were examined. HCT-116 cells were treated with SK-1009, troglitazone and dexamethasone for 2 h, and it was found that only SK-1009 reduced TNFα-stimulated NF-κB transcriptional activity (Suppl. Fig. 2B). After 6 h, dexamethasone tended to reduce TNFα-stimulated NF-κB transcriptional activity (data not shown). These data demonstrated that the effects of SK-1009 on NF-κB transcriptional activity is faster than that of dexamethasone, and suggested that the effect of SK-1009 is not the result of activation of xenobiotic receptor, such as pregnane X receptor.

DISCUSSION

The present study demonstrated that SK-1009 significantly suppresses IL-6 expression levels not only in human macrophage cells, but also in human colon cancer cells stimulated with TNFα. Moreover, we demonstrated clear suppression of activation of transcription factor NF-κB, which has important roles in IL-6 gene expression with SK-1009 treatment.

Many kinds of transcription factors are known to be involved in transcriptional induction of the IL-6 gene, such as NF-κB and NF-IL6. Of these, NF-κB is the major transcription factor responsible for induction of the IL-6 gene expression by LPS and inflammatory cytokines. The NF-κB binding site located between -72 and -63 on the IL-6 gene promoter region is important for IL-6 induction. Both p50 and p65 can bind strongly to the κB-like motif of the IL-6 gene. IL-6 promoter-luciferase reporter gene assay with a site-directed mutant revealed that both NF-IL6 and NF-κB binding sites in the IL-6 gene are required for the synergistic activation. Thus, we speculated that SK-1009 has the potential to inhibit NF-κB transcriptional activity. In addition, SK-1009 suppressed the expression of several inflammation-related genes including...
cytokines, such as TNFα and IL-8 (data not shown), in which NF-κB plays an important role in its induction. These results support the data that NF-κB could be the target of SK-1009. Meanwhile, not all the IL-6 suppressive effect can’t be explained by reduction of NF-κB transcriptional activity, such as that which was observed in RKO cells. We also examined the effects of nuclear receptor activation by SK-1009, but did not obtain data suggesting cross talk between NF-κB transcriptional activity and such nuclear receptors as far as we could find. Further detailed mechanisms underlying suppression of NF-κB transcriptional activity by SK-1009 should be examined in the future.

The importance of NF-κB is demonstrated by its critical role in responses leading to host defense through rapid induction of several genes, such as inflammatory and immunological responses. Dysregulation of NF-κB has been linked to pathological dysregulation of various conditions, including inflammatory reactions, septic shock, acquired immunodeficiency syndrome and cancer. The NF-κB protein exists in the cytoplasm in an inactive state with an inhibitory subunit IκBα. Phosphorylation of residues Ser32 and Ser36 of IκBα and its subsequent degradation allows nuclear translocation of NF-κB. Translocation of NF-κB to the nucleus activates transcription of NF-κB responsive genes, such as IL-6 and TNFα. Thus, inhibition of NF-κB by SK-1009 was suggested to be, at least in part, due to inhibition of degradation of IκBα (Fig. 4). Roles for reactive oxygen species, protein kinases, protein phosphatases, proteases and ceramide have been documented in the pathway of IκBα degradation and NF-κB activation. SK-1009 is so novel a compound that the effects on such reactive oxygen species production, activation of protein kinases, protein phosphatases, proteases and ceramide are not known yet. Further studies need to be carried out to elucidate the effects of SK-1009 on the pathway of inhibition of degradation of IκBα.

Evidence is now accumulating that excessive production of IL-6 is a causative factor for carcinogenesis. Dysregulated activation of NF-κB has also been suggested to be related to promotion of carcinogenesis. Therefore, SK-1009 inhibition of IL-6 production may be causally related to its anticarcinogenic potential. However, it should be borne in mind that NF-κB activity also plays an important role in the immune system. Therefore, it is possible that pronounced inhibition of IL-6 production and transcription factor activities in macrophages and lymphocytes may cause adverse effects, such as immunosuppression and deterioration of the defense system of the host, which may facilitate tumor growth. In order to clarify the applicability of SK-1009 as a cancer preventive agent, animal experiments are now under investigation. Our results have shown an additional candidate NF-κB inhibitor, which could be a cancer preventive agent.
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REFERENCES