BIOCHEMICAL PHARMACOLOGY 76 (2008) 1404–1416

available at www.sciencedirect.com

ScienceDirect

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

SH-5, an AKT inhibitor potentiates apoptosis and inhibits invasion through the suppression of anti-apoptotic, proliferative and metastatic gene products regulated by IκBα kinase activation

Gautam Sethi, Kwang Seok Ahn, Bokyung Sung, Ajaikumar B. Kunnumakkara, Madan M. Chaturvedi, Bharat B. Aggarwal *

Cytokine Research Laboratory, Departments of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, United States

ARTICLE INFO

Article history:
Received 18 March 2008
Accepted 16 May 2008

Keywords:
TNF
AKT
NF-kappaB
Apoptosis
Signal pathway

ABSTRACT

Because the phosphatidylinositol-3-kinase-AKT pathway is emerging as an important regulator of tumor cell survival, inhibitors of this pathway have enormous potential in cancer treatment. A specific inhibitor of AKT, [D-3-deoxy-2-O-methyl-myo-inositol-1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]] (SH-5) has been recently synthesized, but little is known about its effects on cytokine signaling. We found that SH-5 potentiated the apoptosis induced by tumor necrosis factor (TNF), as indicated by intracellular esterase staining, annexin V staining, and caspase-3 activation. This effect of SH-5 correlated with downregulation of various gene products that mediate cell survival, proliferation, metastasis, and invasion, all known to be regulated by NF-κB. SH-5 also blocked NF-κB activation induced by TNF-α, lipopolysaccharide, phorbol ester, and cigarette smoke but not that activated by hydrogen peroxide and RANK ligand, indicating differential requirement of AKT. Inhibition of NF-κB correlated with abrogation of phosphorylation and degradation of IκBα through the inhibition of activation of IκBα kinase (IKK). This led to suppression of the phosphorylation and translocation of p65 and also of NF-κB reporter activity induced by TNFR1, TRADD, TRAF2, NIK, and IKKβ but not that induced by p65 transfection. Thus, our results clearly demonstrate that inhibition of AKT leads to potentiation of apoptosis through modulation of NF-κB signaling.

1. Introduction

AKT, a serine–threonine kinase also known as protein kinase B, is a central signaling molecule in the phosphatidylinositol-3-kinase (PI3K) pathway. [1–3]. This kinase is activated by mitogens and cytokines that function as survival factors. AKT mediates its effects by phosphorylating substrates that decrease the activity of pro-apoptotic proteins or increase the activity of anti-apoptotic proteins [4–7]. Activation of PI3K/AKT signaling results in a disturbance of control of cell proliferation and apoptosis, resulting in competitive growth advantage for tumor cells. Blockade of the PI3K–AKT pathway has been found to sensitize various tumor cell types to apoptotic cell death induced by a variety of chemotherapeutic

* *Corresponding author*. Tel.: +1 713 792 3503/6459; fax: +1 713 794 1613.
E-mail address: aggarwal@mdanderson.org (B.B. Aggarwal).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.05.023

agents [8,9]. Hence, this pathway is an attractive target for the development of novel anticancer strategies. However, the molecular mechanisms for such enhanced induction of tumor cell apoptosis by the combination of a PI3K–AKT inhibitor and anticancer agents have remained largely unknown.

In addition to directly phosphorylating and inactivating pro-apoptotic protein targets, AKT can stimulate signaling pathways that regulate the activity of transcription factor NF-κB (10–16). [10–15]. NF-κB is a family of Rel domain-containing proteins present in the cytoplasm of all cells, where they are kept in an inactive state by a family of anchorin domain-containing proteins, which includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105, and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and IκBα in the cytoplasm; only when activated and translocated to the nucleus is the sequence of events leading to activation initiated. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, H_2O_2, and tumor necrosis factor (TNF), have been shown to activate NF-κB. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and phosphorylation of p65, which in turn leads to the translocation of NF-κB to the nucleus where it binds to specific response elements in the DNA. The phosphorylation of IκBα is catalyzed by IκBα kinase (IKK), which is essential for NF-κB activation by most agents [16–18].

However, the mechanism(s) by which NF-κB-AKT interaction contributes to survival in tumor cells is unknown. In the current study, we used a recently discovered inhibitor of AKT, the phosphatidylinositol ether lipid analogue [D-3-deoxy-2-O-methyl-myo-inositol-1-[(*R*)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]] (SH-5) [19] to investigate the role of NF-κB as a putative mediator of the anti-apoptotic function of AKT in TNF-induced cell signaling. Our results demonstrate that AKT inhibitor potentiates the TNF-induced apoptosis through downregulation of NF-κB-regulated anti-apoptotic gene products and the NF-κB activation pathway.

2. Materials and methods

2.1. Reagents

The phosphatidylinositol ether lipid analogue SH-5 (Fig. 1A) was obtained from Alexis Biochemicals (San Diego, CA, USA). A 50 mM solution of SH-5 was prepared with dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Bacteria-derived human recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA, USA). Cigarette smoke condensate (CSC), prepared as previously described [20], was kindly supplied by Dr. C. Gary Gairola (University of Kentucky, Lexington, KY, USA). Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Invitrogen (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS) and anti-β-actin antibody were obtained from Aldrich–Sigma (St. Louis, MO, USA). N-Acetyl-leucyl-leucyl-norleucinal (ALLN) was purchased from EMD Biosciences, Inc. (San Diego, CA, USA). Antibodies against p65, p50, IκBα, cyclin D1, MMP-9, PARP, IAP1, Bcl-2, Bcl-xL, AKT, and TRAF1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences (San Diego, CA, USA). Phospho-specific anti-IκBα (Ser32/36), and phospho-specific anti-p65 (Ser536) were purchased from Cell Signaling (Beverly, MA, USA). Anti-IKK-α, anti-IKK-β, and phospho-AKT (Ser 473), antibodies were kindly provided by Imgenex (San Diego, CA, USA).

2.2. Cell lines

Cell lines KBM-5 (human myeloid), H1299 (lung adenocarcinoma), and A293 (human embryonic kidney) were obtained from American Type Culture Collection (Manassas, VA, USA). The H1299 cells were cultured in RPMI 1640 medium, the KBM-5 cells were cultured in IMDM medium with 15% FBS, and the A293 cells were cultured in DMEM medium supplemented with 10% FBS. All culture media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Cytotoxicity assay

Cytotoxicity was assayed by the modified tetrazolium salt 3-(4-5-dimethylthiozol-2-yl)2-5-diphenyl-tetrazolium bromide (MTT) assay [21] with following modification. Briefly, the cells (5000 per well) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test samples in a final volume of 0.1 ml for 24 h at 37 °C. Thereafter, 20 μl MTT solution (5 mg/ml in PBS) was added to each well. After a 2-h incubation at 37 °C, 0.1 ml extraction buffer (20% SDS, 50% dimethylformamide) was added; incubation was continued overnight at 37 °C; and then the optical density (OD) at 570 nm was measured by means of a 96-well multiscanner autoreader (Dynatech MR 5000, Chantilly, VA) [23],

2.4. Live/Dead assay

To measure apoptosis, we used the Live/Dead cell viability assay (Invitrogen, Carlsbad, CA), which determines intracellular esterase activity and plasma membrane integrity [22].

2.5. Clonogenic assay

H1299 cells were seeded in six-well plates at 500 cells/well in RPMI 1640 medium containing 10% serum. After 12 h, cells were treated with medium containing indicated concentrations of SH-5 and TNF (1 nM). The medium with SH-5 and TNF was replaced after every 5 days. After 12 days of incubation, colonies were stained with 0.3% crystal violet solution (dissolved in 1:1 mixture of methanol and H_2O) for 2 min, washed once with Dulbecco's phosphate-buffered saline, air-dried, and manually counted. Each point was a mean of three replicate wells.

2.6. Annexin V assay

Annexin V assay was performed as described previously [23].

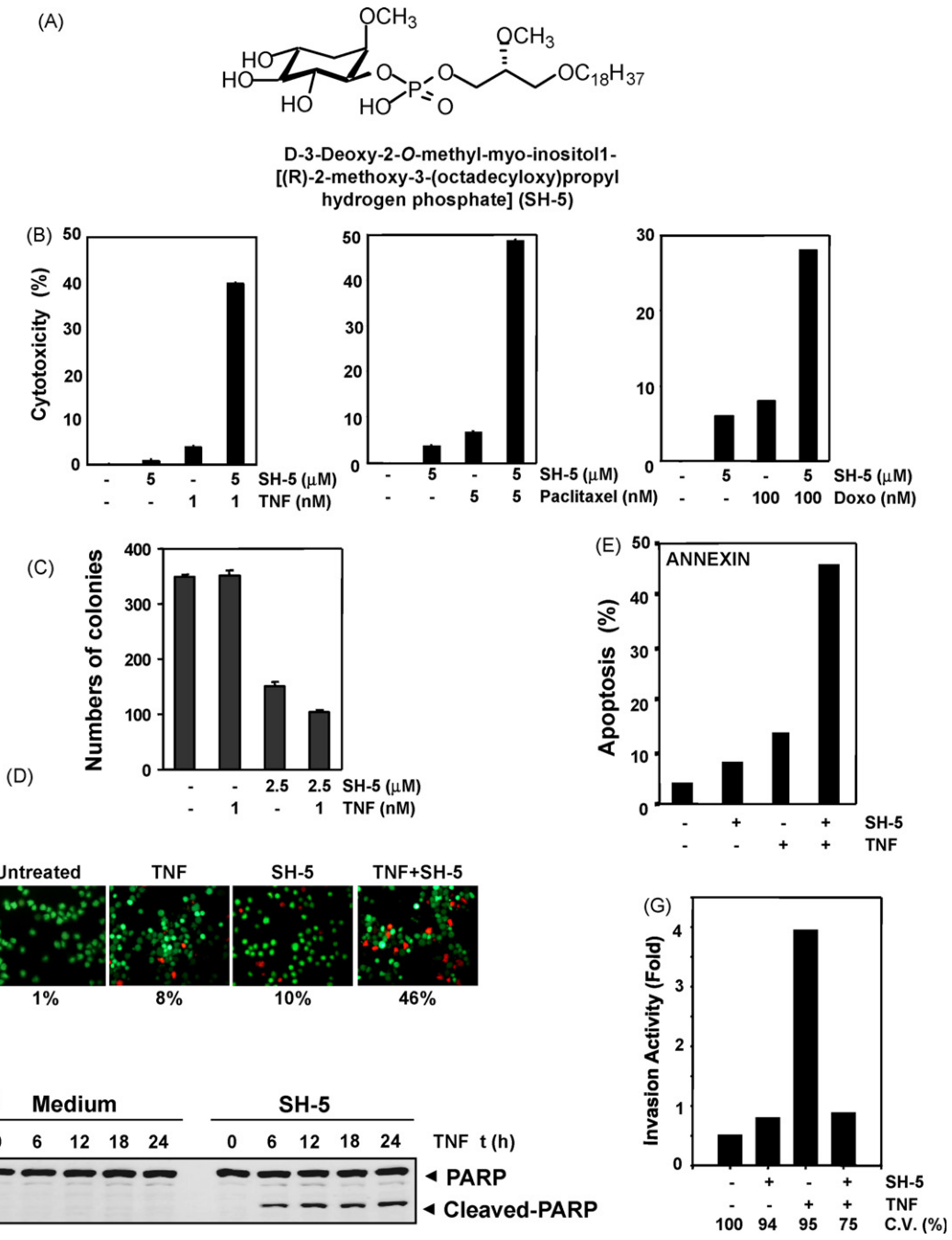

Fig. 1 – (A) The structure of SH-5. (B) SH-5 enhances TNF- and chemotherapeutic agent-induced cytotoxicity. In total, 10,000 cells were seeded in triplicate in 96-well plates. The cells were pretreated with 5 μM SH-5 for 4 h and then incubated with the indicated concentrations of TNF, paclitaxel, and doxorubicin for 24 h. Cell viability was analyzed by the MTT method as described in Section 2. (C) SH-5 potentiates the effect of TNF for growth inhibition of tumor cells H1299 (500 cells/well) were exposed to indicated concentration of SH-5 alone and with 1 nM TNF; and incubated for an additional 12 days before counting colony numbers. Determinations were made in triplicate. (D) SH-5 potentiates TNF-induced apoptosis. KBM-5 cells were pretreated with 5 μM SH-5 for 4 h and then incubated with 1 nM TNF for 16 h. The cells were stained with the Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Section 2. (E) SH-5 enhances TNF-induced annexin V-FITC binding. Cells were pretreated with 5 μM SH-5 for 4 h and then incubated with 1 nM TNF for 16 h. The cells were incubated with a fluorescein isothiocyanate-conjugated annexin V antibody and then analyzed by flow cytometry as described in Section 2. (F) SH-5 potentiates TNF-induced PARP cleavage. Cells were pretreated with 5 μM SH-5 for 4 h and then incubated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using an anti-PARP antibody. (G) SH-5 suppresses TNF-induced invasion activity. H1299 cells (2.5×10^4 cells) were seeded to the top chamber of a Matrigel invasion chamber system overnight in the absence of serum and then treated with 5 μM SH-5 for 4 h. After incubation, the cells were treated with 1 nM TNF in the presence of 1% serum and then assayed for invasion as described in Section 2. Results are expressed as fold activity of the untreated control.

2.7. Invasion assay

The invasion assay was performed using the BD BioCoat tumor invasion system (BD Biosciences, San Jose, CA), as described previously [22]. Briefly, 2.5×10^4 cells were resuspended in serum-free medium and seeded into the upper wells. After incubation overnight, the cells were treated with 5 μM SH-5 for an additional 2 h and then stimulated with 1 nM TNF for 24 h more in the presence of 1% FBS and 5 μM SH-5. The cells that invaded through the Matrigel were labeled with 4 μg/ml calcein acetoxymethylester (Molecular Probes) in PBS for 30 min at 37 °C and subjected to scan fluorescence with a Victor 3 (PerkinElmer Life and Analytical Sciences, Waltham, MA).

2.8. Cyclooxygenase-2 promoter-dependent reporter luciferase gene expression

The COX-2 promoter (−375 to +59; contains one κB binding site), which was amplified from human genomic DNA by using the primers 5′-GAGTCTCTTATTTATTTTT-3′ (sense) and 5′-GCTGCTGAGGAGTTCCTGGACGTGC-3′ (antisense), was kindly provided by Dr. Xiao-Chun Xu (The University of Texas M. D. Anderson Cancer Center, Houston, TX). COX-2 promoter-dependent reporter luciferase gene expression was performed as described previously [23].

2.9. Electrophoretic mobility shift assays (EMSA)

To determine NF-κB activation, we prepared the nuclear extracts and performed EMSA as described previously [24]. The dried gels were visualized, and the radioactive bands were quantified using a Storm820 optical scanner and Imagequant software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

2.10. Western blot analysis

To determine the levels of protein expression, we prepared nuclear, cytoplasmic or whole-cell extracts as described previously [22] and fractionated them by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes, blotted with each antibody, and detected by an ECL regent (GE Healthcare). The bands obtained were quantified using the NIH image software (National Institutes of Health, Bethesda, MD, USA).

2.11. PARP assay

To determine the effect of SH-5 on PARP 40 μg whole-cell extracts were resolved on 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% non-fat milk protein, probed with PARP antibodies (1:3000), and detected by ECL reagent as previously described [25].

2.12. IKK assay

To determine the effect of SH-5 on TNF-induced IKK activation, an IKK assay was performed as described previously [23].

2.13. NF-κB-dependent reporter gene expression assay

To determine the effect of SH-5 on TNF-, TNFR-, TRADD-, TRAF2-, NIK-, IKKβ-, and p65-induced NF-κB-dependent reporter gene transcription, we performed the secretory alkaline phosphatase (SEAP) assay as previously described [23]. Briefly, A293 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected by Fugene6 (Roche Molecular Biochemicals, Mannheim, Germany) with pNF-κB-SEAP (0.25 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.25 μg of the SEAP expression plasmid with 1 μg of the control plasmid, pCMV-FLAG1, for 24 h. We treated the cells for 2 h with 5 μM SH-5 and then stimulated them with 1 nM TNF for further 24 h. To examine the expression levels of various gene-induced reporter genes, the cells were transfected with 0.25 μg of reporter gene plasmid with each 1 μg of expressing plasmid for 24 h and then treated with 5 μM SH-5 for 2 h. The cell culture medium was harvested and analyzed for SEAP activity according to the protocol, essentially in the same way described by the manufacturer (Clontech, Palo Alto, CA, USA) using Victor 3 microplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

2.14. Immunocytochemistry for NF-κB p65 localization

Immunocytochemistry for p65 nuclear localization was performed as described previously [23]. Briefly, the cells were seeded in a chamber slide (Lab-Tek II Chamber Slide System, Nalge Nunc International, Rochester, NY, USA), treated, air-dried, and fixed with 4% paraformaldehyde after permeabilization with 0.2% of Triton X-100. After being washed in PBS, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal antihuman p65 antibody at a 1:200 dilution. After overnight incubation at 4 °C, the slides were again washed, incubated with goat anti-rabbit IgG-Alexa 594 at a 1:200 dilution for 1 h, and the nuclei were counterstained with Hoechst 33342 (50 ng/ml) for 5 min. The stained slides were mounted with a mounting medium purchased from Aldrich–Sigma and analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX, USA) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA, USA).

3. Results

The aim of this study was to investigate the effect of SH-5 on TNF-mediated cellular responses and the NF-κB signaling pathway. Most of our studies were performed using human chronic myeloid leukemia cells (KBM-5) because these cells express both types of TNF receptors. Under the conditions that we used to examine the NF-κB pathway and NF-κB-regulated gene products, SH-5 had no effect on the viability of these cells (data not shown). The structure of SH-5 is shown in Fig. 1A.

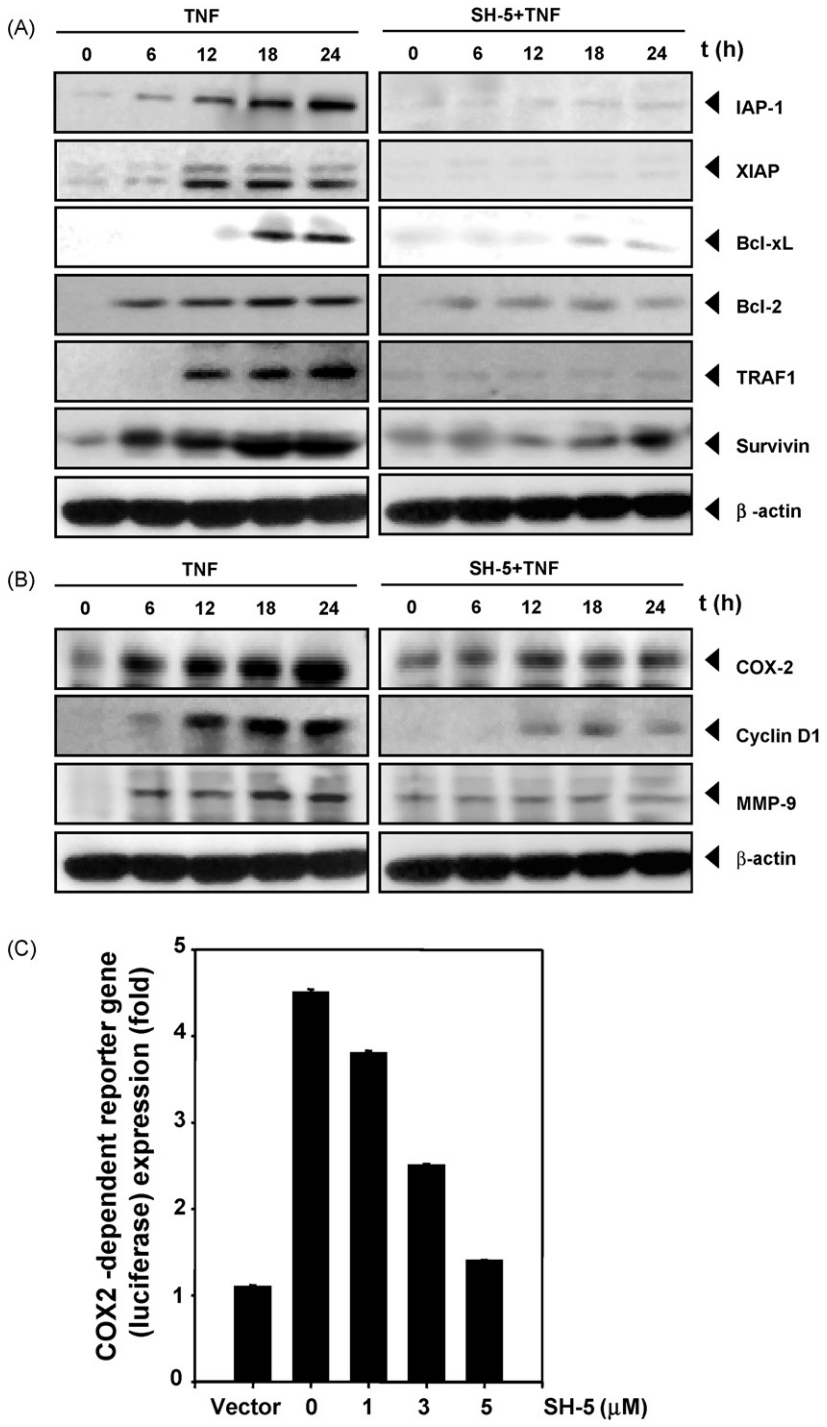

Fig. 2 – SH-5 inhibits TNF-induced expression of NF-κB-dependent gene products associated with anti-apoptosis, metastasis, proliferation and invasion. (A) SH-5 inhibits the expression of TNF-induced anti-apoptotic proteins. KBM-5 cells were incubated with 5 μM SH-5 for 4 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. The same membranes were probed with β-actin antibody to verify equal loading. (B) SH-5 inhibits TNF-induced COX-2, MMP-9, and cyclin D1 expression. KBM-5 cells were incubated with 5 μM SH-5 for 4 h and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. The same membranes were probed with β-actin antibody to verify equal loading. (C) SH-5 inhibits TNF-induced COX-2 promoter activity. H1299 cells were transiently transfected with a COX-2 promoter plasmid linked to the luciferase gene and then treated with the indicated concentrations of SH-5. After 24 h in culture with 1 nM TNF, cells were collected and assayed for luciferase activity as described in Section 2. Results are expressed as fold activity over the activity of the vector control. The results shown are representative of three independent experiments.

3.1. SH-5 potentiates apoptosis induced by TNF and chemotherapeutic agents

NF-κB activation has been shown to suppress apoptosis induced by TNF and chemotherapeutic agents through the expression of gene products regulated by NF-κB [26]. We investigated whether SH-5 modulates the cytotoxic effects of TNF, paclitaxel, and doxorubicin. The effect of SH-5 on TNF- and chemotherapeutic agent-induced apoptosis was examined by the MTT assay. We found that SH-5 significantly enhanced the cytotoxic effects of TNF, paclitaxel, and doxorubicin (Fig. 1B). We also examined whether SH-5 potentiates the effect of TNF by clonogenic assay in H1299 cells. Cells were exposed to the indicated concentrations of SH-5 alone or with TNF, cultured for 12 days, and then counted the number of the colonies. The exposure to SH-5 resulted in dose-dependent reduction in colony formation compared with that of control. TNF enhanced the inhibition of colony formation induced by SH-5 in H1299 (Fig. 1C). These results demonstrate that SH-5 enhances the effect of TNF for inhibition of tumor colony formation. The Live/Dead assay, which measures intracellular esterase activity and plasma membrane integrity, indicated that SH-5 upregulates TNF-induced apoptosis from 8% to 46% (Fig. 1D). The results of annexin V staining, which examines early apoptosis, also showed that TNF-induced apoptosis was enhanced by incubation with SH-5 (Fig. 1E). When we examined the cells for caspase-mediated PARP cleavage, we found that the SH-5 enhanced apoptosis induced by TNF (Fig. 1F). Together, these results support the conclusion that SH-5 potentiates the apoptotic effect of TNF and chemotherapeutic agents.

3.2. SH-5 suppresses TNF-induced tumor cell invasion activity

NF-κB activation also plays an important role in tumor cell invasion [27]. Whether SH-5 can modulate TNF-induced invasive activity was investigated *in vitro*. For this study, we seeded the tumor cells into the upper wells of a Matrigel invasion chamber in the absence of serum. The cells were pretreated with SH-5 and then treated with TNF in the presence of 1% serum. As shown in Fig. 1G, TNF induced invasion activity by almost four-fold, and SH-5 suppressed this activity.

3.3. SH-5 represses TNF-induced NF-κB-dependent anti-apoptotic gene products

NF-κB regulates the expression of the anti-apoptotic proteins IAP-1, XIAP, Bcl-2, Bcl-xL, TRAF1, and survivin [16]. We investigated whether SH-5 can modulate the expression of these anti-apoptotic gene products. We found that TNF induced the expression of these anti-apoptotic proteins in a time-dependent manner, and SH-5 blocked it (Fig. 2A).

3.4. SH-5 represses the TNF-induced NF-κB-dependent gene products involved in the proliferation, metastasis, and invasion of tumor cells

We also investigated whether SH-5 can modulate NF-κB-regulated gene products involved in the proliferation, metastasis and invasion of tumor cells. TNF has been shown to induce COX-2, cyclin D1, and MMP-9, all of which have NF-κB binding sites in their promoters [16]. We therefore investigated whether SH-5 inhibits the TNF-induced expression of these proteins. Cells untreated with SH-5 and those pretreated with SH-5 were examined for TNF-induced gene products by Western blot analysis using specific antibodies (Fig. 2B). TNF induced the expression of COX-2, MMP-9, and cyclin D1 in a time-dependent manner, and SH-5 abolished the expression of these proteins.

3.5. SH-5 represses TNF-induced cyclooxygenase-2 promoter activity

We next determined whether SH-5 affected COX-2 promoter activity, which is regulated by NF-κB [16]. As shown in Fig. 2C,

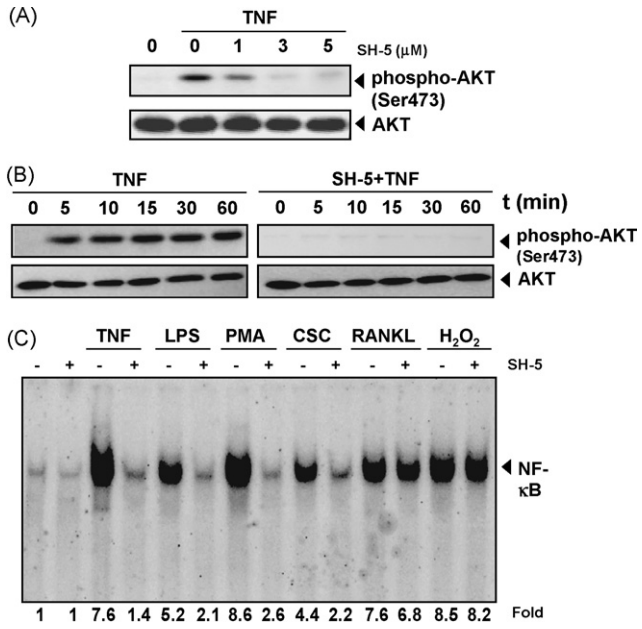

Fig. 3 – (A) SH-5 suppresses AKT activation induced by TNF in KBM-5 cells. KBM-5 cells were incubated with the indicated concentrations of SH-5 for 4 h and treated with 1 nM TNF for 30 min. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. (B) KBM-5 cells were preincubated with 5 μM SH-5 for the indicated times and then treated with 1 nM TNF for 30 min. Whole-cell extracts were prepared and analyzed by Western blotting using phospho-AKT antibody. The same membranes were probed with non-phosphorylated AKT antibody to verify equal loading. (C) SH-5 differentially inhibits NF-κB activation induced by TNF, LPS, PMA, CSC, H_2O_2 and RANKL. Human chronic myeloid leukemia KBM-5 cells were preincubated with 5 μM SH-5 for 4 h and then treated with 0.1 nM TNF for 30 min, 25 ng/ml PMA for 2 h, 10 μg/ml LPS for 2 h and 10 μg/ml CSC for 1 h, 500 μM H_2O_2 for 2 h, and 10 nM RANKL for 60 min. Nuclear extracts were analyzed for NF-κB activation as described in Section 2. The results shown are representative of three independent experiments.

SH-5 inhibited TNF-induced COX-2 promoter activity in a dose-dependent manner.

3.6. SH-5 inhibits TNF-induced AKT activation in KBM-5 cells

We determined the dose and time of exposure to SH-5 required to suppress AKT activation. Western blot results showed that SH-5 inhibited TNF-mediated AKT activation in a dose-dependent manner (Fig. 3A). However, it alone had no effect on AKT activation. The suppression of AKT activation by SH-5 was also found to be time dependent (Fig. 3B). The level of non-phosphorylated AKT remained unchanged in both cases (Fig. 3A and B, lower panels).

3.7. SH-5 differentially inhibits NF-κB activation induced by carcinogens and inflammatory stimuli

TNF, LPS, CSC, PMA, RANK ligand (RANKL), and H_2O_2 activate NF-κB but by different mechanisms [20,26,28]. Therefore, we examined the effect of SH-5 on the activation of NF-κB by these agents. Pretreatment of cells with SH-5 suppressed the activation of NF-κB induced by TNF, LPS, CSC, and PMA but did not affect NF-κB activation induced by RANKL or H_2O_2 (Fig. 3C). These results suggest that AKT activation is not involved in the NF-κB activation pathway induced by RANK ligand and H_2O_2.

3.8. SH-5 suppresses NF-κB activation in a dose- and time-dependent manner

We next investigated the dose and time of exposure to SH-5 required to suppress NF-κB activation in KBM-5 cells. EMSA results showed that SH-5 alone had no effect on NF-κB activation. However, it inhibited TNF-mediated NF-κB activation in a dose-dependent manner (Fig. 4A). The suppression of NF-κB activation by SH-5 was also found to be time dependent (Fig. 4B). When nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF-κB) and the p65 (RelA) subunits of NF-κB, the resulting bands were shifted to higher molecular masses (data not shown), suggesting that the TNF-activated complex consisted of p50 and p65 (data not shown).

3.9. Inhibition of NF-κB activation by SH-5 is not cell type specific

Distinct signal transduction pathways can mediate NF-κB induction in different cell types [29], so we investigated whether

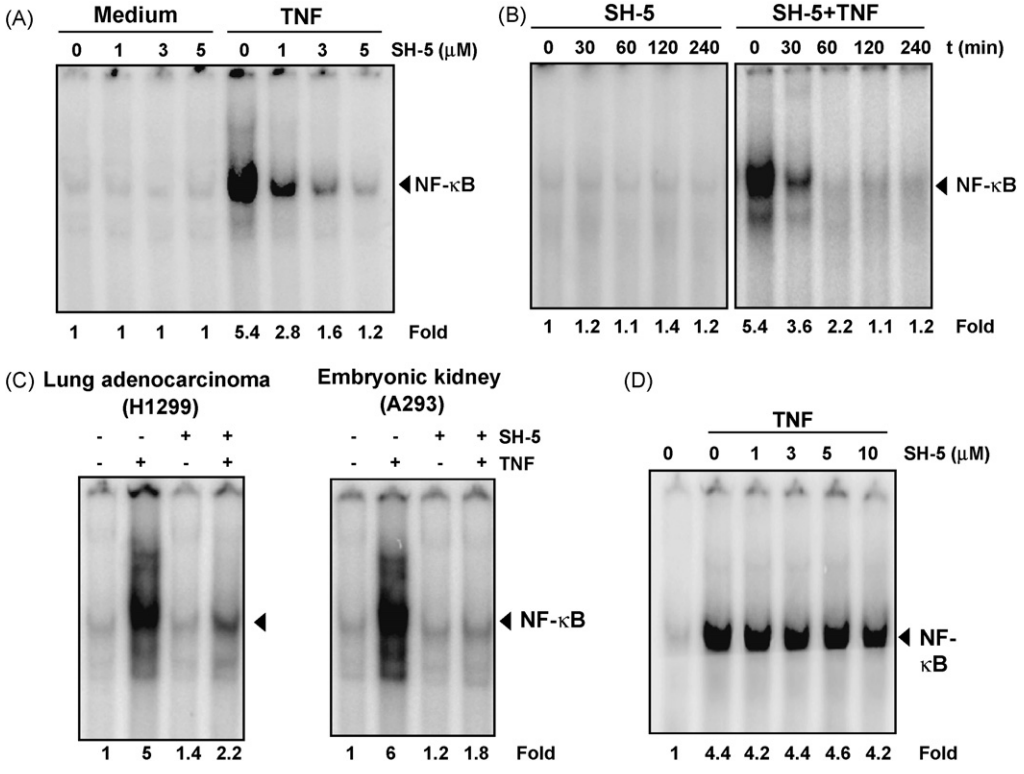

Fig. 4 – (A) SH-5 suppresses NF-κB activation by dose dependent manner. KBM-5 cells were incubated with the indicated concentrations SH-5 for 4 h and treated with 0.1 nM TNF for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. (B) SH-5 suppresses NF-κB activation by time dependent manner, KBM-5 cells were preincubated with 5 μM SH-5 for the indicated times and then treated with 0.1 nM TNF for 30 min. The nuclear extracts were prepared and assayed for NF-κB activation by EMSA. (C) SH-5 suppresses TNF-induced NF-κB activation in different cell types. Human lung carcinoma H1299 and human embryonic kidney A293 cells were incubated with 5 μM SH-5 for 4 h, followed by incubation with 0.1 nM TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. (D) The direct effect of SH-5 on NF-κB complex was investigated. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nM TNF and incubated for 30 min with the indicated concentrations of SH-5. They were then assayed for NF-κB activation by EMSA.

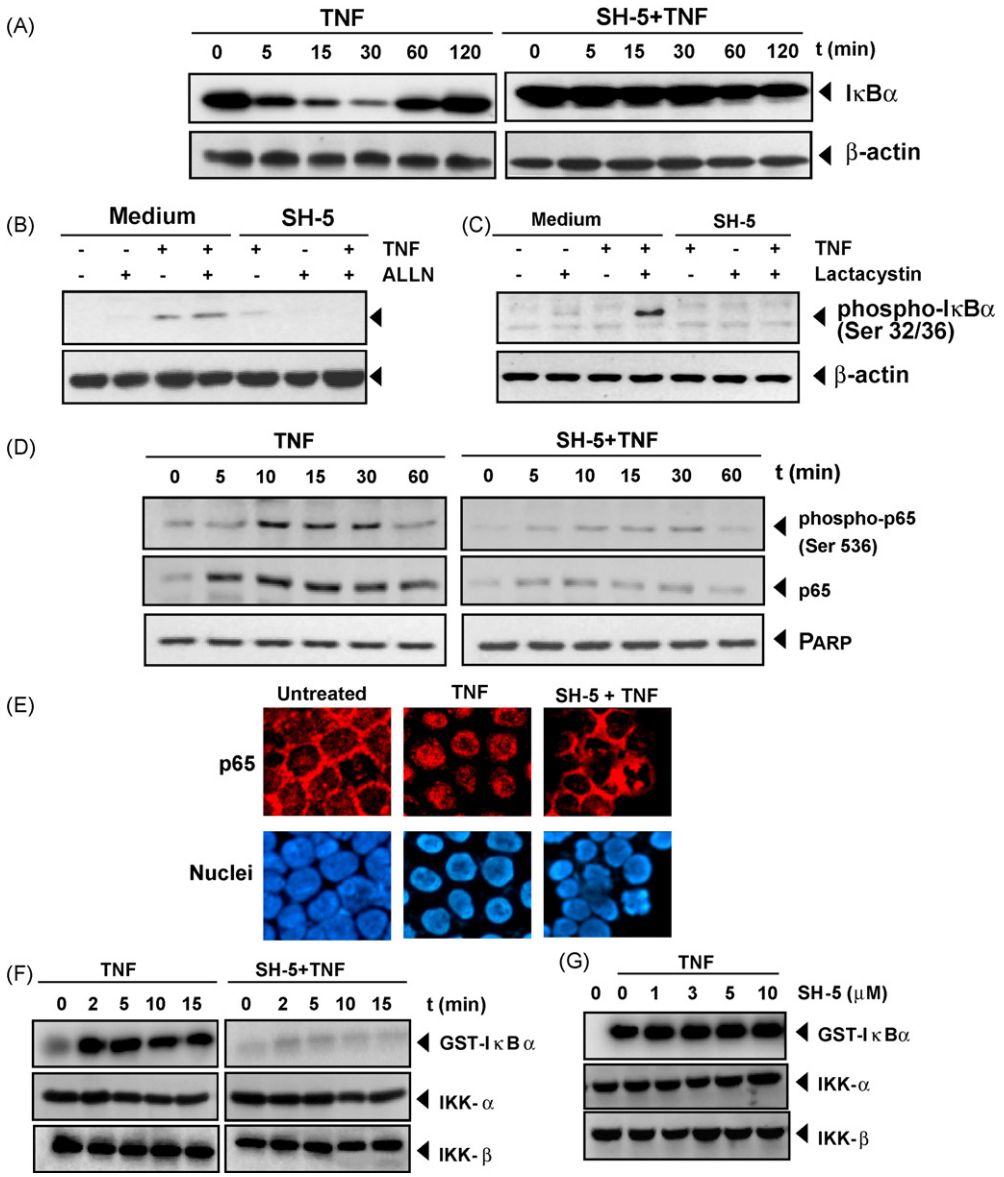

Fig. 5 – (A) SH-5 inhibits TNF-induced degradation of IκBα. KBM-5 cells were incubated with 5 μM SH-5 for 4 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared and analyzed by Western blotting using antibodies against anti-IκBα and β-actin. (B) Effect of SH-5 on the phosphorylation by IκBα by TNF. Cells were preincubated with 5 μM SH-5 for 4 h, incubated with 50 μg/ml N-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific IκBα antibody. The same membranes were reprobed with β-actin antibody. (C) Effect of SH-5 on the phosphorylation by IκBα by TNF. Cells were preincubated with 5 μM SH-5 for 4 h, incubated with 20 μM lactacystin for 30 min, and then treated with 0.1 nM TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific IκBα antibody. The same membranes were reprobed with β-actin antibody. (D) SH-5 inhibits TNF-induced phosphorylation and translocation of p65. KBM-5 cells were either untreated or pretreated with 5 μM SH-5 for 4 h at 37 °C and then treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against phospho-specific p65 and p65. (E) SH-5 inhibits TNF-induced nuclear translocation of p65. KBM-5 cells were first treated with SH-5 for 4 h at 37 °C and then exposed to 1 nM TNF for 20 min. After cytospin, immunocytochemical analysis was performed as described in Section 2. (F) The effect of SH-5 on the activation of IKK by TNF was investigated. KBM-5 cells were incubated with 5 μM SH-5 for 4 h, and then treated with 1 nM TNF for different time intervals. Whole-cell extracts (500 μg) were prepared and immunoprecipitated with antibodies against IKK-α. Thereafter, the immune complex kinase assay was performed as described in Section 2. To examine the effect of SH-5 on the level of expression of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. (G) The direct effect of SH-5 on the activation of IKK induced by TNF was investigated. Whole-cell extracts (500 μg) were prepared from 1 nM TNF treated cells and then immunoprecipitated with antibodies against IKK-α and IKK-β. The immunocomplex kinase assay was performed after incubation with indicated concentrations of SH-5 for 30 min.

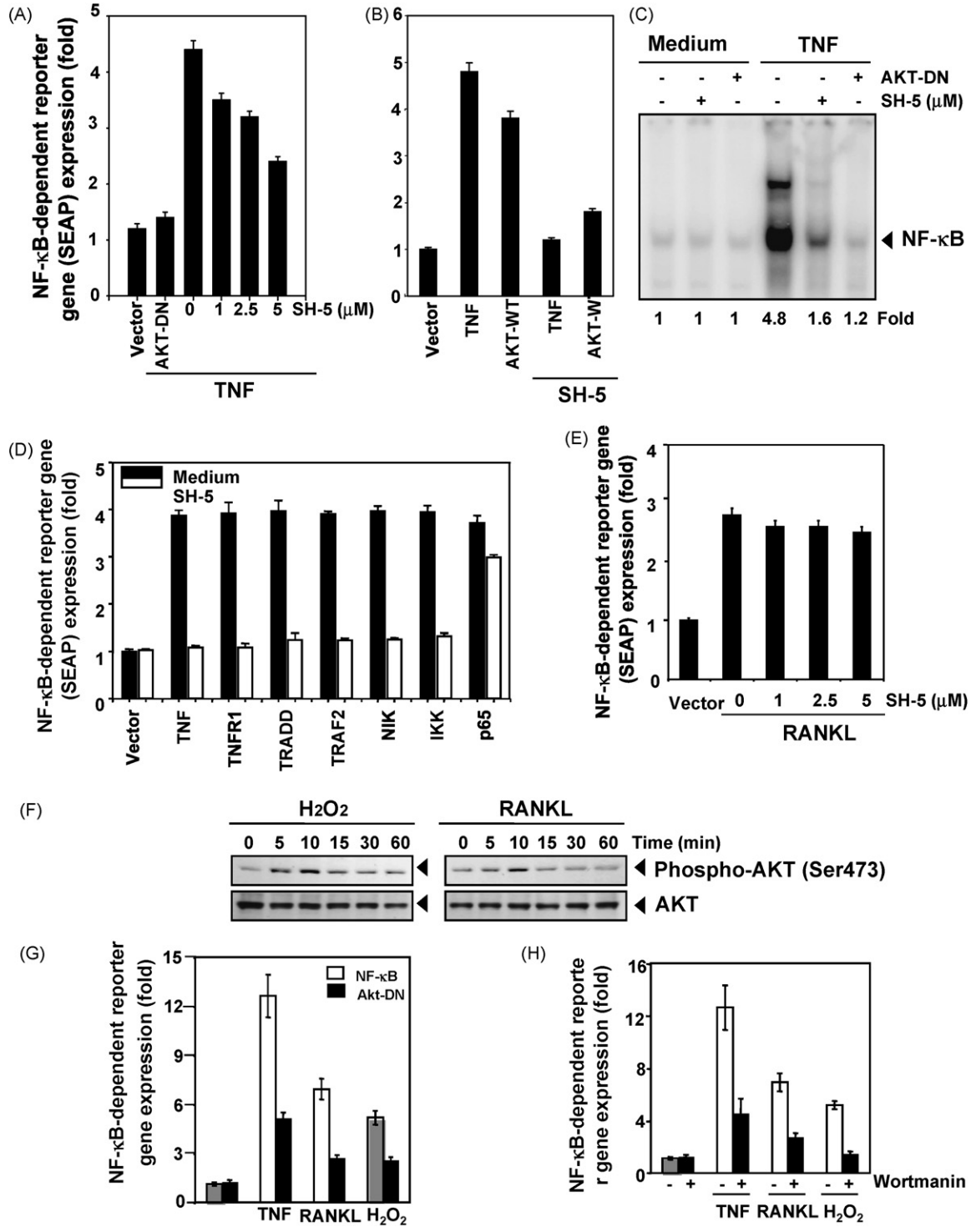

Fig. 6 – (A) SH-5 and AKT-DN inhibit TNF-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with a NF-κB-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of SH-5 or co-transfected with dominant negative AKT (AKT-DN) gene plasmid along with NF-κB-SEAP containing plasmid. After 24 h in culture with 1 nM TNF, cell supernatants were collected and assayed for SEAP activity. Results are expressed as fold activity over the activity of the vector control. (B) Human embryonic kidney A293 cells were transiently transfected with an AKT gene plasmid, and then treated with 5 μM SH-5 for 4 h. After transfection/treatment, the cells were treated with 1 nM TNF for 24 h. After 24 h in culture with 1 nM TNF, cell supernatants were collected and assayed for SEAP activity. (C) Human embryonic kidney A293 cells were transiently transfected with a AKT-DN plasmid for 24 h and/or treated with 5 μM SH-5 for 4 h. After transfection/treatment, the cells were treated with 0.1 nM TNF for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. (D) SH-5 inhibits NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, and IKK-β but not by p65. Cells were transiently transfected with

SH-5 could block TNF-induced NF-κB activation in human small cell lung carcinoma H1299 and human embryonic kidney A293 cells. TNF activated NF-κB in both cell types, and SH-5 completely inhibited the activation (Fig. 4C). These results indicated that there was a lack of cell type specificity.

3.10. SH-5 does not directly affect binding of NF-κB to the DNA

Some NF-κB inhibitors, including N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; the serine protease inhibitor), herbimycin A (protein tyrosine kinase inhibitor), caffeic acid phenethyl ester (CAPE), and plumbagin, directly modify NF-κB to suppress its DNA binding. We investigated whether SH-5 mediates its effect through a similar mechanism. EMSA showed that SH-5 did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells (Fig. 4D). These results suggest that SH-5 inhibits NF-κB activation by a mechanism different from that of TPCK, herbimycin A, or CAPE.

3.11. SH-5 inhibits TNF-induced IκBα degradation

Because IκBα degradation is required for activation of NF-κB [30], we determined whether SH-5's inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation. We found that TNF-induced IκBα degradation in control cells at 15 min, but in SH-5-pretreated cells TNF had no effect on IκBα degradation (Fig. 5A).

3.12. SH-5 inhibits TNF-dependent IκBα phosphorylation

To determine whether the inhibition of TNF-induced IκBα degradation was due to an inhibition of IκBα phosphorylation, we used the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) to block degradation of IκBα. Cells were pretreated with SH-5, treated with ALLN for 30 min, exposed to TNF, and then examined for IκBα phosphorylation status by Western blot analysis using an antibody that recognizes the serine-phosphorylated form of IκBα. SH-5 completely suppressed the IκBα phosphorylation induced by TNF in the presence of the proteasome inhibitor (Fig. 5B). The other proteasome inhibitor, lactacystin, showed similar result to ALLN on IκBα phosphorylation induced by TNF (Fig. 5C).

3.13. SH-5 inhibits TNF-induced phosphorylation of p65

TNF induces the phosphorylation of p65, which is required for its transcriptional activity [31]. After phosphorylation, the p65 subunit is translocated to the nucleus. In the nuclear fraction from the TNF-treated cells, there was a time-dependent increase in the phosphorylated form of p65, and SH-5 treatment suppressed the phosphorylation (Fig. 5D).

3.14. SH-5 inhibits TNF-induced nuclear translocation of p65

We performed immunocytochemical analysis to determine whether SH-5 can inhibit TNF-induced nuclear translocation of p65. The results showed that SH-5 significantly inhibited TNF-induced p65 translocation to the nucleus (Fig. 5E).

3.15. SH-5 inhibits TNF-induced IκBα kinase activation

IKK activation is required for the phosphorylation of IκBα [30]. Because SH-5 inhibits the phosphorylation and degradation of IκBα, we tested the effect of SH-5 on TNF-induced IKK activation. As shown in Fig. 5F, SH-5 completely suppressed TNF-induced activation of IKK. Neither TNF nor SH-5 had any effect on the expression of IKK-α or IKK-β proteins.

To evaluate whether SH-5 suppresses IKK activity directly by binding to IKK or indirectly by suppressing its activation, we incubated whole-cell extracts from untreated cells and TNF-stimulated cells with anti-IKK-α and IKK-β antibodies. After precipitation with protein A/G-agarose beads, the immunocomplex was treated with various concentrations of SH-5. Results from the immune complex kinase assay showed that SH-5 did not directly affect the activity of IKK. This finding suggests that SH-5 modulates TNF-induced IKK activation (Fig. 5G).

3.16. SH-5 represses TNF-induced NF-κB-dependent reporter gene expression

As DNA binding alone does not always correlate with NF-κB-dependent gene transcription [32], we also investigated the effect of SH-5 on TNF-induced reporter gene transcription. We found that TNF activated the transcription of the NF-κB reporter gene and that transfection with AKT-DN and SH-5 treatment completely inhibited it in a dose-dependent manner (Fig. 6A).

an NF-κB-containing SEAP reporter gene plasmid alone or with the indicated plasmids. After transfection, the cells were treated with 5 μM SH-5 for 4 h. TNF-treated cells were treated with 1 nM TNF for an additional 24 h. After 24 h in culture with TNF, cell supernatants were collected and assayed for SEAP activity. (E) SH-5 had no effect on RANKL-induced NF-κB-dependent reporter gene expression. A293 cells were transiently transfected with a NF-κB-SEAP containing plasmid and then treated with the indicated concentrations of SH-5. After 24 h in culture with 10 nM RANKL, cell supernatants were collected and assayed for SEAP activity as described in Section 2. (F) H_2O_2 and RANKL activated AKT. A293 cells were incubated with 500 μM of H_2O_2 or 10 nM RANKL for indicated times, whole-cell extracts were prepared, and phospho-AKT was detected by Western blot as described in Section 2. The same blots were stripped and reprobed with AKT antibody to verify equal protein loading. (G) A293 cells were transiently transfected with a NF-κB-SEAP reporter plasmid with an AKT-DN gene plasmid gene and then treated with 1 nM TNF, 500 μM H_2O_2 or 10 nM RANKL. After 24 h in culture with stimuli, cell supernatants were collected and assayed for SEAP activity. (H) SH-5 has no effect on H_2O_2 or RANKL-induced NF-κB activation. A293 cells were transiently transfected with a NF-κB-SEAP reporter plasmid. After transfection, the cells treated with 5 μM SH-5 for 2 h, and then incubated with 1 nM TNF, 500 μM H_2O_2 or 10 nM RANKL for an additional 24 h. The cell supernatants were collected and assayed for SEAP activity as described in Section 2. The results shown are representative of three independent experiments.

SH-5 also substantially inhibited NF-κB-dependent SEAP expression in cells transfected with AKT wild-type plasmid (Fig. 6B). Transfection with the AKT-DN plasmid also significantly suppressed TNF-induced NF-κB activation as measured by DNA binding in human embryonic kidney A293 cells (Fig. 6C). TNF-induced NF-κB activation is mediated through the sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK, leading to the degradation of IκBα and p65 nuclear translocation [33]. Thus, we also investigated where in the pathway SH-5 suppresses gene transcription. To determine this, cells were transfected with TNFR1-, TRADD-, TRAF2-, NIK-, IKK-β-, and p65 plasmids, along with the NF-κB-regulated SEAP reporter construct, incubated with SH-5, and then monitored for NF-κB-dependent SEAP expression. SH-5 suppressed the NF-κB reporter activity induced by the TNFR1-, TRADD-, TRAF2-, NIK-, and IKK-β plasmids but had no effect on the activity induced by the p65 plasmid (Fig. 6D). These results suggest that SH-5 affects a step upstream of p65.

3.17. *SH-5 did not affect RANKL-induced NF-κB-dependent reporter gene expression*

Because SH-5 failed to suppress RANKL-induced NF-κB DNA binding, we also investigated its effect on RANKL-induced reporter gene transcription. We transiently co-transfected the cells with the NF-κB-regulated SEAP reporter construct, incubated them with SH-5, and then stimulated them with RANKL. We found that RANKL activated the transcription of the NF-κB reporter gene and that transfection with different doses of SH-5 did not significantly affect the gene transcription (Fig. 6E).

3.18. *H_2O_2 and RANKL induced AKT activation in A293 cells*

We further examined whether H_2O_2 and RANKL can induce AKT activation in A293 cells. A293 cells were incubated with H_2O_2 or RANKL for indicated time and whole-cell extracts were prepared and examined for phosphorylated AKT by Western blot analysis with antibody that recognizes AKT phosphorylated at Ser 473. As shown in Fig. 6F, both H_2O_2 and RANKL activated AKT in A293 cells in within 5–10 min.

3.19. *Effects of AKT-DN on H_2O_2 and RANKL-induced NF-κB-dependent reporter gene expression*

Since AKT-DN abrogated TNF-induced NF-κB DNA binding, we also investigated its effect on RANKL or H_2O_2-induced NF-κB activation using reporter gene assay. We transiently co-transfected the cells with the NF-κB-regulated SEAP reporter and AKT-DN constructs, and then stimulated them with RANKL or H_2O_2. We found that deficiency of AKT failed to induce NF-κB activation (Fig. 6G).

3.20. *Wortmanin inhibits TNF, RANKL and H_2O_2-induced NF-κB-dependent reporter gene expression*

We investigated the effect of other AKT inhibitor on H_2O_2 and RANKL-induced reporter gene transcription. We transiently transfected cells with the NF-κB-regulated SEAP reporter plasmid, treated them with wortmanin (100 nM) for 2 h, and then induced NF-κB activation with, TNF, H_2O_2 and RANKL. We found that wortmanin suppressed TNF, RANKL and H_2O_2-induced NF-κB activation (Fig. 6H).

4. Discussion

In this study, we investigated the role of SH-5 on TNF-mediated cellular responses and the TNF-induced NF-κB activation pathway. We found that SH-5 potentiated the apoptosis induced by TNF. This effect of SH-5 correlated with downregulation of various gene products that mediate cell survival, proliferation, metastasis, and invasion all known to be regulated by NF-κB. We found that this AKT inhibitor suppressed the activation of NF-κB induced by TNF, LPS, cigarette smoke, and PMA but did not affect NF-κB activation induced by RANK ligand or H_2O_2. NF-κB inhibition correlated with suppression of IKK activation, IκBα phosphorylation and degradation, p65 phosphorylation and nuclear translocation, and inhibition of NF-κB-dependent reporter gene expression.

We found for the first time that SH-5 potentiates TNF-induced apoptosis in chronic myeloid leukemia cells. When we sought to investigate the mechanism of this potentiation, we found that SH-5 downregulated the expression of various anti-apoptotic gene products (IAP1, XIAP, Bcl-2, Bcl-xL, TRAF, and survivin). We also found that inhibition of AKT downregulated the expression of COX-2, cyclin D1, and MMP-9. COX-2 also has been implicated in carcinogenic processes, and its overexpression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate anti-apoptotic cellular defenses, and augment immunologic resistance through the production of prostaglandin E_2 [34]. The downregulation of MMP-9 correlated with the inhibition of TNF-induced invasion by SH-5. MMP-9 plays a crucial role in tumor invasion and angiogenesis by mediating the degradation of the extracellular matrix, and the inhibition of MMP activity has been shown to suppress lung metastasis [35]. Lu and Wahl [36] recently showed that AKT plays an important role in MMP-9 production in monocytes. In addition to COX-2 and MMP-9, SH-5 also suppressed the production of TNF-α in titanium particle induced murine monocyte, RAW 264.7 cells, through inhibition of PI3K–AKT signaling pathway [37].

This is first report to suggest that AKT is needed for NF-κB activation induced by TNF, LPS, PMA, and CSC. However, we found that AKT is not needed for NF-κB activation induced by RANKL or H_2O_2 in myeloid leukemia cells. Our results differ from those of a recent report that found that NF-κB activation in endothelial cells by TNF is AKT independent [10]. This difference may be due to cell type specificity. Although we did not examine endothelial cells, our results show that AKT was needed for NF-κB activation by TNF, irrespective of the cell type. Our results are in agreement with those of other reports that have suggested that AKT is involved in the activation of NF-κB in response to TNF-α [11,12], IL-1β [13], PMA [14], PDGF [15], and pervanadate [38]. It has been reported that AKT is activated by both RANKL [39,40] and H_2O_2 [41]. Why RANKL and H_2O_2-induced AKT activation does not lead to NF-κB activation is not clear. Our results are in agreement with a

previous report that wortmannin, a PI3-K inhibitor, has no effect on H_2O_2-induced NF-κB activation [42].

In response to most of these stimuli, NF-κB activation requires the activation of IKK. The suppression of TNF-induced IKK activation by SH-5 suggests that it abolishes NF-κB activation by other agents through a suppression of IKK activation. This result is in agreement with previous reports indicating that the role of AKT in inducing NF-κB occurs through IKK-dependent degradation of IκBα [14,15,38]. However, most of these reports suggest that AKT directly phosphorylates IKKα. Gene deletion experiments, however, indicate that IKKα plays small role in TNF-induced NF-κB activation. The role of IKKα has been linked to the non-canonical pathway of NF-κB activation [43]. Therefore, it seems likely that AKT is part of the complex that activates IKK, and in normal cells, in addition to its role in an alternate pathway, it is also required for activation of NF-κB by the canonical pathway. Whether AKT associates transiently to this IKK kinase complex is not clear at present. We observed that suppression of IKK inhibited IκBα phosphorylation and degradation. We also found SH-5 suppressed NF-κB reporter activity induced by TNF and the activity following transfection with wild-type AKT plasmid. These results are in agreement with those of Sizemore et al. [13], who showed that both IKKα and IKKβ are required AKT-mediated p65 phosphorylation and NF-κB activation in response to TNF and IL-1β. Our results are also consistent with those of Kane et al. [14], who reported that kinase-deficient forms of both IKKα and IKKβ inhibited NF-κB reporter activity induced by AKT. Our results are consistent with those of another report, which showed that AKT requires IKKβ to upregulate the transactivation domain of the p65 subunit of NF-κB [44].

We also found that AKT is needed for NF-κB reporter gene expression induced by TNFR1, TRADD, TRAF2, NIK, and IKKβ. However, p65-induced NF-κB activation was unaffected by AKT inhibitor. These results suggested that the SH-5 acts at a step upstream from p65. Thus these results indicate that AKT is needed for IKK activation but not for the transactivation potential of p65. Overall our results indicate that the suppression of NF-κB activation plays a critical role in potentiation of apoptosis by SH-5. Our results also demonstrate the critical role of AKT in expression of gene products involved in cell survival, proliferation, inflammation, and invasion.

Acknowledgements

We would like to thank Michael Worley for carefully editing the manuscript and providing valuable comments and Dr. Bryant Darnay for RANKL protein. Dr. Aggarwal is Ransom Horne, Jr., Professor of Cancer Research. This work was supported by a grant from the Clayton Foundation for Research (to B. B. A.) and National Institutes of Health PO1 grant CA91844 on lung chemoprevention (to B.B.A).

REFERENCES

[1] Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 1997;387:673–6.
[2] Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. Proc Natl Acad Sci USA 2001;98:10983–5.
[3] Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 2001;26:657–64.
[4] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–41.
[5] Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu S, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96: 857–68.
[6] Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;282:1318–21.
[7] Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, Glassford J, et al. Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1. J Biol Chem 2000;275:21960–8.
[8] Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 2005;5:921–9.
[9] Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002;14:381–95.
[10] Madge LA, Pober JS. A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells. J Biol Chem 2000;275:15458–65.
[11] Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999;401:82–5.
[12] Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin Jr AS, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol 2000;20:1626–38.
[13] Sizemore N, Leung S, Stark GR. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 1999;19:4798–805.
[14] Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 1999;9:601–4.
[15] Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86–90.
[16] Aggarwal BB. Nuclear factor-kappaB: the enemy within. Cancer Cell 2004;6:203–8.
[17] Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev 2004;18:2195–224.
[18] Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 1997;91:243–52.
[19] Kozikowski AP, Sun H, Brognard J, Dennis PA. Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. J Am Chem Soc 2003;125:1144–5.
[20] Anto RJ, Mukhopadhyay A, Shishodia S, Gairola CG, Aggarwal BB. Cigarette smoke condensate activates nuclear transcription factor-kappaB through phosphorylation and degradation of IkappaB(alpha): correlation with induction of cyclooxygenase-2. Carcinogenesis 2002;23:1511–8.
[21] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[22] Sethi G, Ahn KS, Pandey MK, Aggarwal BB. Celastrol, a novel triterpene, potentiates TNF-induced apoptosis and suppresses invasion of tumor cells by inhibiting

NF-kappaB-regulated gene products and TAK1-mediated NF-kappaB activation. Blood 2007;109:2727–35.
[23] Sethi G, Ahn KS, Sandur SK, Lin X, Chaturvedi MM, Aggarwal BB. Indirubin enhances tumor necrosis factor-induced apoptosis through modulation of nuclear factor-kappa B signaling pathway. J Biol Chem 2006;281:23425–3.
[24] Chaturvedi MM, Mukhopadhyay A, Aggarwal BB. Assay for redox-sensitive transcription factor. Methods Enzymol 2000;319:585–602.
[25] Anto RJ, Mukhopadhyay A, Denning K, Aggarwal BB. Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl-2 and Bcl-xl. Carcinogenesis 2002;23:143–50.
[26] Garg A, Aggarwal BB. Nuclear transcription factor-kappaB as a target for cancer drug development. Leukemia 2002;16:1053–68.
[27] Liotta LA, Thorgeirsson UP, Garbisa S. Role of collagenases in tumor cell invasion. Cancer Metastasis Rev 1982;1:277–88.
[28] Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999;18:6853–66.
[29] Bonizzi G, Piette J, Merville MP, Bours V. Distinct signal transduction pathways mediate nuclear factor-kappaB induction by IL-1beta in epithelial and lymphoid cells. J Immunol 1997;159:5264–72.
[30] Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2002;109(Suppl.):S81–96.
[31] Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[32] Campbell KJ, Rocha S, Perkins ND. Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. Mol Cell 2004;13:853–65.
[33] Simeonidis S, Stauber D, Chen G, Hendrickson WA, Thanos D. Mechanisms by which IkappaB proteins control NF-kappaB activity. Proc Natl Acad Sci USA 1999;96:49–54.
[34] Shiraga M, Yano S, Yamamoto A, Ogawa H, Goto H, Miki T, et al. Organ heterogeneity of host-derived matrix metalloproteinase expression and its involvement in multiple-organ metastasis by lung cancer cell lines. Cancer Res 2002;62:5967–73.
[35] Hirschowitz E, Hidalgo G, Doherty D. Induction of cyclo-oxygenase-2 in non-small cell lung cancer cells by infection with DeltaE1, DeltaE3 recombinant adenovirus vectors. Gene Ther 2002;9:81–4.
[36] Lu Y, Wahl LM. Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKKalpha/NF-kappaB pathway. J Leukoc Biol 2005;78:259–65.
[37] Smith MV, Lee MJ, Islam AS, Rohrer JL, Goldberg VM, Beidelschies MA, et al. Inhibition of the PI3K–Akt signaling pathway reduces tumor necrosis factor-alpha production in response to titanium particles in vitro. J Bone Joint Surg Am 2007;89:1019–27.
[38] Beraud C, Henzel WJ, Baeuerle PA. Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF-kappaB activation. Proc Natl Acad Sci USA 1999;96:429–34.
[39] Wong BR, Besser D, Kim N, Arron JR, Vologodskaia M, Hanafusa H, et al. TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol Cell 1999;4:1041–9.
[40] Gingery A, Bradley E, Shaw A, Oursler MJ. Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. J Cell Biochem 2003;89:165–79.
[41] Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J 2003;22:5501–10.
[42] Manna SK, Aggarwal BB. Wortmannin inhibits activation of nuclear transcription factors NF-kappaB and activated protein-1 induced by lipopolysaccharide and phorbol ester. FEBS Lett 2000;473:113–8.
[43] Gustin JA, Ozes ON, Akca H, Pincheira R, Mayo LD, Li Q, et al. Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. J Biol Chem 2004;279:1615–20.
[44] Meng F, Liu L, Chin PC, D'Mello SR. Akt is a downstream target of NF-kappa B. J Biol Chem 2002;277:29674–80.