

Signaling and Regulation

Sesamin Manifests Chemopreventive Effects through the Suppression of NF- κ B-Regulated Cell Survival, Proliferation, Invasion, and Angiogenic Gene Products

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Abstract

Agents that are safe, affordable, and efficacious are urgently needed for the prevention of chronic diseases such as cancer. Sesamin, a lipid-soluble lignan, is one such agent that belongs to a class of phytoestrogens, isolated from sesame (*Sesamum indicum*), and has been linked with prevention of hyperlipidemia, hypertension, and carcinogenesis through an unknown mechanism. Because the transcription factor NF- κ B has been associated with inflammation, carcinogenesis, tumor cell survival, proliferation, invasion, and angiogenesis of cancer, we postulated that sesamin might mediate its effect through the modulation of the NF- κ B pathway. We found that sesamin inhibited the proliferation of a wide variety of tumor cells including leukemia, multiple myeloma, and cancers of the colon, prostate, breast, pancreas, and lung. Sesamin also potentiated tumor necrosis factor- α -induced apoptosis and this correlated with the suppression of gene products linked to cell survival (e.g., Bcl-2 and survivin), proliferation (e.g., cyclin D1), inflammation (e.g., cyclooxygenase-2), invasion (e.g., matrix metalloproteinase-9, intercellular adhesion molecule 1), and angiogenesis (e.g., vascular endothelial growth factor). Sesamin downregulated constitutive and inducible NF- κ B activation induced by various inflammatory stimuli and carcinogens, and inhibited the degradation of I κ B α , the inhibitor of NF- κ B, through the suppression of phosphorylation of I κ B α and inhibition of activation of I κ B α protein kinase, thus resulting in the suppression of p65 phosphorylation and nuclear translocation, and NF- κ B-mediated reporter gene transcription. The inhibition of I κ B α protein kinase activation was found to be mediated through the inhibition of TAK1 kinase. Overall, our results showed that sesamin may have potential against cancer and other chronic diseases through the suppression of a pathway linked to the NF- κ B signaling. *Mol Cancer Res*; 8(5); 751–61. ©2010 AACR.

Introduction

Most modern drugs, commonly called targeted therapies, designed within last two decades for cancer are not so safe, are highly ineffective, and are unaffordable. Thus, agents that can overcome these problems are needed not only for treatment but also for the prevention of cancer. “Let food be thy medicine or medicine be thy food” proclaimed by Hippocrates about 25 centuries ago, or 12

serving of fruits and vegetables daily, proclaimed recently by the National Cancer Institute, suggests to look for agents in the diet that may have potential for cancer. Sesamin, a class of phytoestrogens, is one such agent isolated from the oil of sesame seeds (*Sesamum indicum*), which has been shown to exhibit variety of activities (1). Sesame contains water-soluble lignan glycosides (sesaminol triglucoside and sesaminol diglucoside) and lipid-soluble lignans (sesamin and sesamol). Sesamin, a furofuran class of lignin, has been found to reduce hypertension (2), lowers serum and hepatic cholesterol (3–5) and decreases serum triglycerides (6), inhibits endotoxin-mediated shock (7), and suppress 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis (8). These effects of sesamin were shown to be due to its ability to inhibit δ 5 desaturase involved in polyunsaturated fatty acid synthesis (9); decrease arachidonic acid and prostaglandin synthesis (8, 10); increase serum levels of γ -tocopherol (11); diminish the endotoxin-induced interleukin (IL)-1 β , prostaglandin E2 (PGE2), and thromboxane B2 production (7); and abrogate the production of IL-6 (10).

In cell culture, sesamin has been shown to inhibit the growth of a variety of tumor cells including leukemia

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(12, 13) and gastric cancer (14). Mechanism by which sesamin mediates anticancer effects are not fully understood. However, its role in the suppression of reactive oxygen species and mitogen-activated protein kinase activation (15), inhibition of nitric oxide production (16), inflammatory cytokine production (17), and inhibition of expression of cyclin D1 in human cancer cells (18) have been reported.

Because inflammation, survival, proliferation, chemosensitization, carcinogenesis, invasion, and angiogenesis all have been linked to the transcription factor NF- κ B-regulated gene products, we postulated that sesamin must mediate its effects through modulation of the NF- κ B activation pathway. This factor belongs to Rel family and is activated by a wide array of stimuli, which include various types of carcinogens, inflammatory stimuli, and growth factors (19). Aberrant activation of NF- κ B cascade has been linked to the expression of genes that are linked with survival, proliferation, invasion, and metastasis of tumors (20).

Thus, in the present report, we investigated how sesamin modulates NF- κ B-mediated cellular responses, gene products, and signaling pathway. We show that sesamin suppresses the proliferation of a wide variety of tumor cells; downregulates the expression of gene products that mediate inflammation, tumor cell survival, cell proliferation, cell invasion, and angiogenesis; and abrogates both constitutive and inducible NF- κ B activation pathway stimulated by various carcinogens and inflammatory stimuli through the inhibition of activation of I κ B α protein kinase (IKK).

Materials and Methods

Reagents. A 50-mmol/L solution of sesamin was prepared initially in DMSO, stored as small aliquots at -20°C , and then thawed and diluted in a cell culture medium as required. Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech. Penicillin, streptomycin, RPMI 1640, Iscove's modified Dulbecco's medium, and DMEM were obtained from Invitrogen. Fetal bovine serum (FBS) was supplied by Atlanta Biologicals. Antibodies against p65, p50, I κ B α , cyclin D1, cyclooxygenase-2 (COX-2), Bcl-2, and intercellular adhesion molecule 1 (ICAM-1) were obtained from Santa Cruz Biotechnology. For immunocytochemistry, an antibody against p65 was obtained from Abcam. An anti-vascular endothelial growth factor (VEGF) antibody was purchased from Thermo Scientific. Phosphospecific anti-I κ B α (Ser^{32/36}) and phosphospecific anti-p65 (Ser⁵³⁶) antibodies were purchased from Cell Signaling Technology. Anti-IKK- α and anti-IKK- β antibodies were provided by Imgenex.

Cell lines. The cell lines KBM-5 (human chronic myeloid leukemia), A293 (human embryonic kidney carcinoma), H1299 (human lung adenocarcinoma), HCT116 (human epithelial colon cancer), and RPMI-8226 (human multiple myeloma) were obtained from the American Type Culture Collection. KBM-5 cells were cultured in Iscove's

modified Dulbecco's medium with 15% fetal bovine serum; H1299 and RPMI-8226 cells were cultured in RPMI 1640; and A293 and HCT116 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Culture media were supplemented with 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin.

Electrophoretic mobility shift assay. To assess NF- κ B activation, nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was done as previously described (21). Briefly, nuclear extracts prepared from treated cells and nontreated cells ($1 \times 10^6/\text{mL}$) were incubated with ^{32}P -end-labeled 45-mer double-stranded oligonucleotide (15 μg protein with 16 fmol DNA) from the HIV long terminal repeat, 5'-TTGTTACAAGGGACTTTCCGCTGGGAC-TTCCAGGGAGGCGTGG-3 (boldface indicates NF- κ B binding sites), for 30 minutes at 37°C . The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized with a Storm 820 and radioactive bands were quantified using the ImageQuant software (GE Healthcare).

Western blot analysis. Western blot analysis was done as previously described (22). Briefly, 30 μg protein were resolved on SDS-PAGE and probed with specific antibodies according to the manufacturer's recommended protocol. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 2 hours, and finally detected by enhanced chemiluminescence reagent (GE Healthcare). The bands were quantified with a Personal Densitometer Scan v1.30 using the ImageQuant software version 3.3 (GE Healthcare).

IKK assay. IKK assay was done as previously described (22). Briefly, the IKK complex from whole-cell extracts was precipitated with IKK- α and then pulled down with protein A/G-agarose beads (Pierce). After 2 hours, the beads were washed with lysis buffer and resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl_2 , 2 mmol/L DTT, 20 mCi [γ - ^{32}P] ATP, 10 mmol/L unlabeled ATP, and 2 μg substrate glutathione *S*-transferase-I κ B α (amino acids 1-54) and incubated at 30°C for 30 minutes. Boiling with SDS sample buffer for 5 minutes terminated the reaction. Finally, the protein was resolved on 10% SDS-PAGE; the gel was dried; and the radioactive bands were visualized with a Storm 820. To determine the total amounts of IKK- α and IKK- β in each sample, 30 μg of whole-cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK- α or anti-IKK- β antibodies.

Immunocytochemical analysis for NF- κ B p65 localization. To determine the effect of sesamin on TNF-induced nuclear translocation of p65, we used an immunocytochemical method. Slides were analyzed under a fluorescence microscope (Labophot-2; Nikon) and images were captured using a Photometrics Coolsnap CF color camera (Nikon) as previously described (22).

NF- κ B-dependent reporter gene expression assay. An NF- κ B-dependent reporter gene expression assay was done as previously described (23). The effect of sesamin on

TNF-, TNF receptor (TNFR)-, TNFR-associated death domain (TRADD)-, NF- κ B-inducing kinase (NIK)-, and transforming growth factor β -activated kinase 1 (TAK1)/TAK-1 binding protein-1 (TAB1)-dependent reporter gene expression was analyzed using a secretory alkaline phosphatase (SEAP) assay.

Cell proliferation assay. The tumor cell proliferation was evaluated using a modified tetrazolium salt MTT assay. Briefly, cells (2000 cells/well) were incubated in the presence or absence of the indicated concentration of sesamin for 0, 2, 4, and 6 days. Thereafter, 20 μ L of MTT solution (5 mg/mL in PBS) were added to each well. After 2 hours of incubation at 37°C, 0.1 mL of the extraction buffer (20% SDS, 50% dimethyl formamide) was added. After incubation overnight at 37°C, the optical densities at 570 nm were measured using a 96-well plate reader.

Live/Dead assay. The Live/Dead assay (Invitrogen), which assesses plasma membrane integrity, was used to measure the intracellular esterase activity. This assay was done as previously described (24).

Results

Sesamin is a lignan with a structure as shown (Fig. 1A). The aim of the present study is to determine the effect of this lignan on NF- κ B-mediated cellular responses, NF- κ B-regulated gene products, and on the signaling pathway leading to NF- κ B activation. Most of our studies were done on KBM-5 cells as these cells express both types of TNFRs. TNF was used for most studies because of a variety of reasons; first, TNF is a primary mediator of inflammation; second, TNF activates both apoptosis and NF- κ B; third, TNF-induced NF- κ B activation cascade is well characterized; and fourth, TNF-induced NF- κ B can downregulate apoptosis. Under the conditions we examined the effect of sesamin on NF- κ B pathway, it had no effect on cell viability; thus, downregulation of NF- κ B was not due to decrease in viability.

Sesamin suppresses cell proliferation in various tumor cells. We examined whether sesamin can modulate the proliferation of various tumor cell types. As shown in Fig. 1B, sesamin by itself suppressed the proliferation of human leukemic cells (such as KBM-5 and K562) and multiple myeloma cells (U266). The suppression was both dose and time dependent. Besides these cells, sesamin also inhibited the proliferation of solid tumor cells, such as human pancreatic cancer MiaPaCa-2 cells, human colon cancer HCT 116 cells, human prostate cancer DU145, human lung adenocarcinoma H1299, and human breast cancer MDA-MB-231 cells (Fig. 1B). The 50% inhibitory sesamin dose was found to be 42.7, 48.3, 51.7, 60.2, 57.2, 58.3, 40.1, and 51.1 μ mol/L for KBM-5, K562, U266, DU145, HCT116, MiaPaCa-2, H1299, and MDA-MB-231 cells, respectively.

Sesamin potentiated TNF-induced apoptosis. We sought to determine whether sesamin affects TNF-induced apoptosis in human chronic myeloid leukemia KBM-5 cells. Using a Live/Dead assay, which measures intracellular

esterase activity and assesses plasma membrane integrity, we found that sesamin increased the TNF-induced apoptosis from 9% to 65% in KBM-5 cells (Fig. 1C, top).

To determine whether the effect is cell type specific, we also examined the effect of sesamin on TNF-induced apoptosis in human multiple myeloma U266 cells. Like KBM-5 cells, sesamin also increased the TNF-induced apoptosis from 10% to 47% in U266 cells (Fig. 1C, bottom).

Sesamin inhibited TNF-induced cell survival gene products. How sesamin potentiates the apoptotic effects of TNF was investigated. One of the possible mechanisms is through the downregulation of cell survival gene products, such as Bcl-2 and survivin. We found that TNF induced the expression of Bcl-2 and survivin and sesamin inhibited this expression (Fig. 2A).

Sesamin inhibited the TNF-induced expression of cell-proliferative gene products. Both cyclin D1 and COX-2 have been linked with the proliferation of different types of tumor cells. Thus, we investigated the effect of sesamin on the expression of cyclin D1 and COX-2 induced by TNF treatment. We found that TNF induced the expression of these gene products and that pretreatment with sesamin inhibited this expression (Fig. 2B).

Sesamin inhibited the expression of gene products involved in invasion and angiogenesis. TNF also induces the expression of genes involved in adhesion (e.g., ICAM-1) and invasion [matrix metalloproteinase-9 (MMP-9)]. Whether sesamin affects the expression of ICAM-1 and MMP-9 was examined. As shown in Fig. 2C, TNF induced the expression ICAM-1 and MMP-9, and sesamin suppressed the expression.

VEGF plays a major role in the process of tumor angiogenesis (25). We found that TNF induced expression of VEGF and sesamin inhibited the expression (Fig. 2C).

Sesamin inhibited the NF- κ B activation induced by TNF in a dose- and time-dependent manner. All the cellular response and gene products modulated by sesamin as described above are regulated by NF- κ B activation. Whether sesamin can suppress NF- κ B activation was examined directly. For this, we exposed KBM-5 cells to sesamin at different concentrations and then exposed them to TNF and examined for the activation of NF- κ B. We found that sesamin by itself had no effect on the activation of NF- κ B. However, it suppressed the TNF-induced NF- κ B activation in a dose-dependent manner, with maximum inhibition occurring at a concentration of 100 μ mol/L (Fig. 3A, left). Moreover, when we assessed the cell viability under these conditions, it was >90%.

Whether the suppression of NF- κ B by sesamin was time dependent was also examined. For this, KBM-5 cells were treated with sesamin for different time intervals followed by 30-minute exposure to TNF. We observed that sesamin inhibited the activation of NF- κ B triggered by TNF in a time-dependent manner, with optimum inhibition occurring at 12 hours (Fig. 3A, right).

Sesamin inhibited NF- κ B activation induced by carcinogens and other inflammatory stimuli. Earlier studies reported from our laboratory and by others clearly

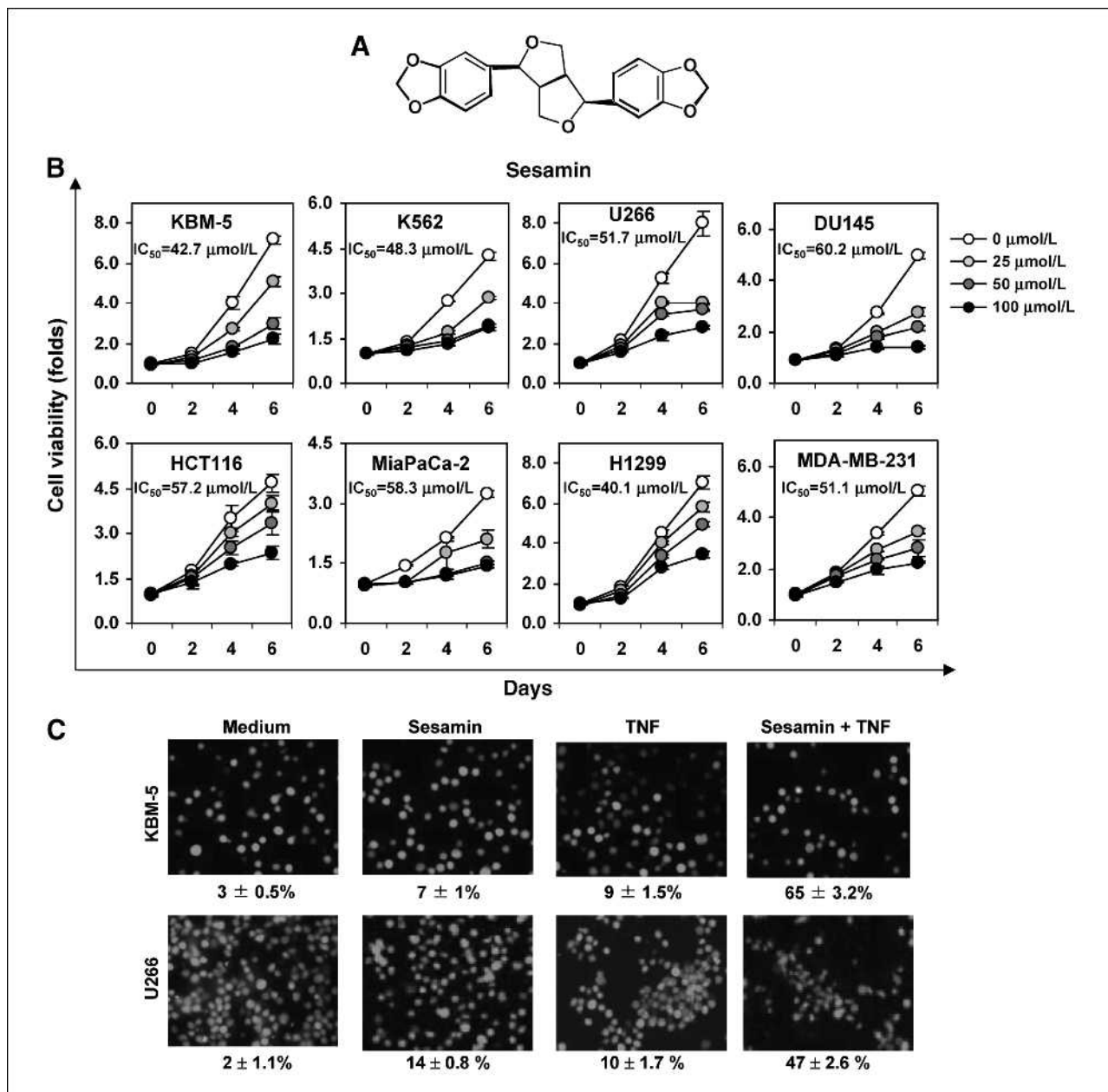


FIGURE 1. A, chemical structure of sesamin. B, sesamin suppresses tumor cell proliferation. Cells were incubated with 0, 25, 50, and 100 $\mu\text{mol/L}$ sesamin for different days. Cell proliferation was then analyzed by the MTT method as described under Materials and Methods. C, sesamin potentiates TNF-induced cytotoxicity. KBM-5 (1×10^5) or U266 (1×10^5) cells were incubated with 100 $\mu\text{mol/L}$ sesamin for 12 h and then incubated with 1 nmol/L TNF for 24 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described under Materials and Methods.

showed that a wide variety of agents, which include cigarette smoke condensate, tumor promoters such as okadaic acid, phorbol myristate acetate, and inflammatory agents such as hydrogen peroxide and lipopolysaccharide (LPS), can activate NF- κB but the mechanisms by which these agents induce activation of NF- κB vary significantly (22, 26). Whether sesamin affects NF- κB activation induced by all these agents was examined. As shown in Fig. 3B, sesamin suppressed the activation NF- κB in-

duced by all these agents. So it can be concluded that this lignan acts at a step in the NF- κB activation pathway that is common to all of these agents.

Sesamin does not have any effect on the binding of NF- κB p65 subunit to the DNA. We also investigated whether this lignan can directly interact with the p65 subunit of NF- κB and inhibit its binding to DNA. Nuclear extracts isolated from TNF-treated KBM-5 cells were exposed to sesamin at different concentrations and then

examined for binding to DNA. As given in Fig. 3C, we found that sesamin did not modulate the p65 binding to DNA even at the highest dose.

Sesamin inhibited constitutive NF- κ B expression.

A wide variety of tumor cell types are known to harbor constitutively active form of NF- κ B, which often results in chemoresistance and treatment failure (19). Multiple myeloma cell lines (e.g., RPMI-8226) are known to express

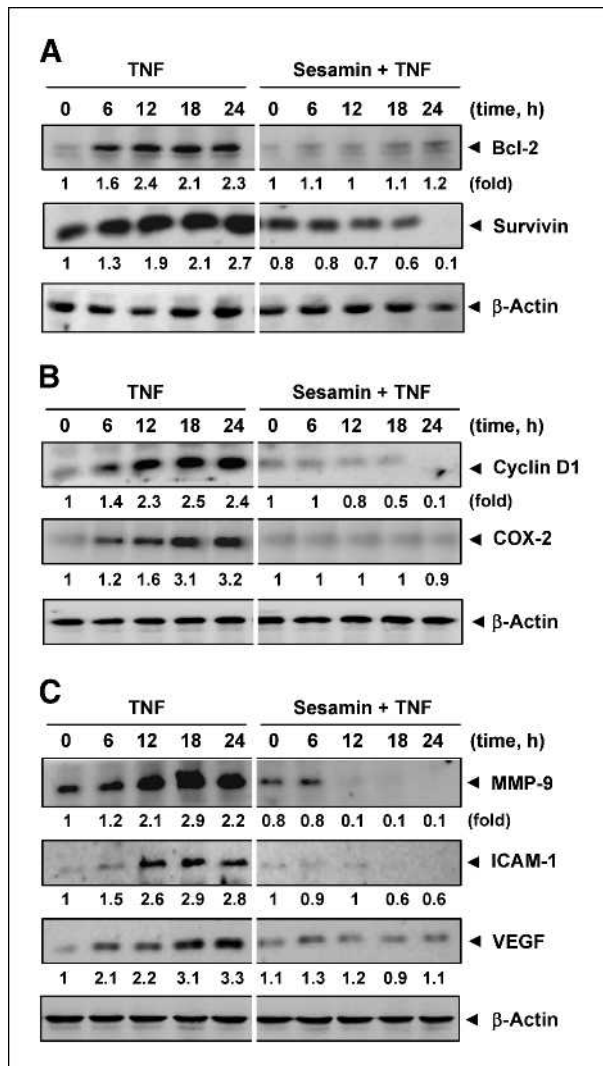


FIGURE 2. A, sesamin suppresses the expression of TNF-induced tumor cell survival proteins. KBM-5 cells were incubated with 100 μ M/L sesamin for 12 h and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. B and C, sesamin inhibits the expression of tumor cell-proliferative and metastatic proteins. KBM-5 cells were incubated with 100 μ M/L sesamin for 12 h and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the indicated antibodies. Anti- β -actin antibody was used as a loading control. Densitometric values of bands were corrected based on β -actin and were expressed relative to that of nontreated cells, which was set as 1.0.

constitutively active NF- κ B (27). Whether sesamin affects NF- κ B expression in these cells was examined. For this, we exposed cells to sesamin at different concentrations for 12 hours and then analyzed them for DNA binding by EMSA. We found that this lignan completely suppressed constitutive NF- κ B activation in RPMI-8226 cells (Fig. 3D, left), indicating that this sesamin can suppress both inducible as well as constitutive NF- κ B activation.

Inhibition of NF- κ B activation by sesamin is not cell type specific. Our next aim was to examine whether the inhibition of activation of NF- κ B by sesamin is specific to a particular cell type. We treated human lung adenocarcinoma H1299 cell and human colon cancer cell line HCT116 to sesamin and then exposed to TNF. EMSA showed that this lignan inhibited the activation of NF- κ B, indicating that the effect is not cell type specific (Fig. 3D, right).

Sesamin inhibits TNF-dependent phosphorylation and degradation of I κ B α . Translocation of NF- κ B to the nucleus is accompanied by phosphorylation, ubiquitination, and degradation of I κ B α , the inhibitory subunit associated with the NF- κ B complex (28). To determine whether the inhibition of TNF-induced NF- κ B activation is associated with the degradation of I κ B α , we pretreated KBM-5 cells with sesamin and then exposed them to TNF at various time intervals. We analyzed nuclear extracts for NF- κ B activation using EMSA and cytoplasmic extracts for I κ B α degradation using Western blotting. TNF activated NF- κ B in a time-dependent manner; however, in sesamin-pretreated cells, we found that there is no activation of NF- κ B (Fig. 4A).

Western blot analysis showed that TNF-induced I κ B α degradation started at 5 minutes after TNF treatment and reached maximum level at 10 minutes. The resynthesis of I κ B α started at 60 minutes after TNF exposure (Fig. 4B, left). However, we noticed no degradation of I κ B α in sesamin-pretreated cells (Fig. 4B, right). These results indicated that sesamin mediates its effect through the suppression of TNF-induced I κ B α degradation, which in turn leads to the suppression of the activation of NF- κ B.

We also decided to determine whether the inhibition of TNF-induced degradation of I κ B α was caused by the inhibition of phosphorylation of I κ B α . For this purpose, we used the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) to block this degradation. We performed a Western blot analysis using an antibody that specifically recognized the I κ B α phosphorylated at serine 32. The results of this analysis showed that TNF induced I κ B α phosphorylation at serine 32 and 36, and that sesamin suppressed this phosphorylation (Fig. 4C).

Sesamin inhibits IKK activation induced by TNF. TNF-induced activation of the enzyme IKK that triggers the phosphorylation of I κ B α (28). We sought to determine whether in KBM-5 cells sesamin inhibits TNF-induced activation of IKK using immune complex assays. As given in Fig. 4D, TNF activated IKK in a time-dependent manner and sesamin suppressed the TNF-induced activation of IKK. Neither TNF nor sesamin affected the expression of IKK- α (Fig. 4D, left).

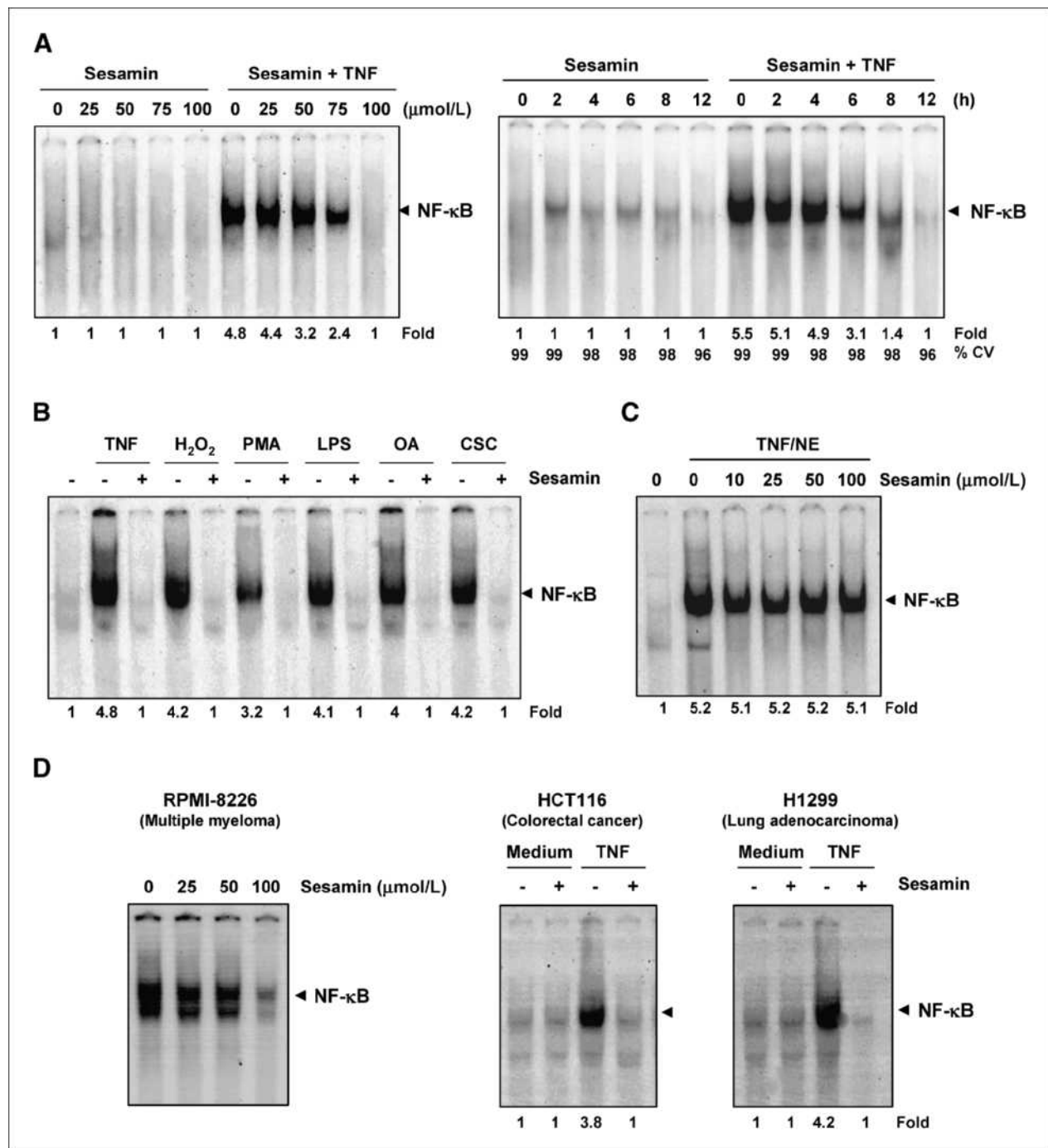


FIGURE 3. A, dose- and time-dependent effect of sesamin on TNF-induced NF-κB activation. Left, KBM-5 cells were incubated with the indicated concentrations of sesamin for 12 h and treated with 0.1 nmol/L TNF for 30 min. Right, KBM-5 cells were preincubated with 100 μmol/L sesamin for the indicated time points and then treated with 0.1 nmol/L TNF for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. B, sesamin inhibits NF-κB activation induced by cigarette smoke condensate (CSC), H₂O₂, phorbol myristate acetate (PMA), LPS, okadaic acid, and TNF. KBM-5 cells were preincubated with 100 μmol/L sesamin for 12 h and then treated with 0.1 nmol/L TNF for 30 min, 250 μmol/L H₂O₂ for 2 h, 25 ng/mL phorbol myristate acetate for 2 h, 10 μg/mL LPS for 1 h, 500 nmol/L okadaic acid for 4 h, and 10 μg/mL cigarette smoke condensate for 2 h. Nuclear extracts were analyzed for NF-κB activation by EMSA. C, sesamin did not inhibit direct binding of NF-κB to DNA. Nuclear extracts were prepared from nontreated KBM-5 cells or cells treated with 0.1 nmol/L TNF for 30 min, incubated for 30 min with indicated concentrations of sesamin for 30 min, and EMSA was performed. D, effect of sesamin on constitutive and inducible NF-κB activation. Left, Human multiple myeloma cells (RPMI-8226) were incubated with the indicated concentrations of sesamin for 12 h, the nuclear extracts were prepared and analyzed for NF-κB activation by EMSA. Right, human colorectal cancer cells (HCT116) and human lung adenocarcinoma cells (H1299) were pretreated with 100 μmol/L sesamin for 12 h, treated with 0.1 nmol/L TNF for 30 min, and then EMSA was performed.

We then decided to examine whether sesamin suppresses IKK activity directly by binding to IKK or indirectly by suppressing its activation. For this, we incubated the immune complexes with various concentrations of sesamin and then examined the IKK activity *in vitro*. The results showed that sesamin did not directly inhibit the activity of IKK (Fig. 4D, right).

TNF-induced translocation of NF- κ B p65 subunit to nucleus is inhibited by sesamin. Once the degradation of I κ B α started, it triggers the nuclear translocation of p65 subunit. We decided to determine whether sesamin has any effect on the TNF-induced nuclear translocation of

p65 subunit. Immunocytochemical analysis showed that sesamin suppressed the TNF-induced translocation of p65 subunit to the nucleus in KBM-5 cells (Fig. 5A). However, in nontreated cells and cells pretreated with sesamin, p65 was localized in the cytoplasm, whereas in cells treated with TNF alone, p65 subunit was translocated to the nucleus. These results support the conclusion that sesamin inhibits nuclear translocation of p65.

Sesamin suppressed the TNF-induced phosphorylation of NF- κ B p65 subunit. It is now established that transcriptional activation of p65 requires phosphorylation at the serine 536 residues (29). Thus, we also sought to investigate

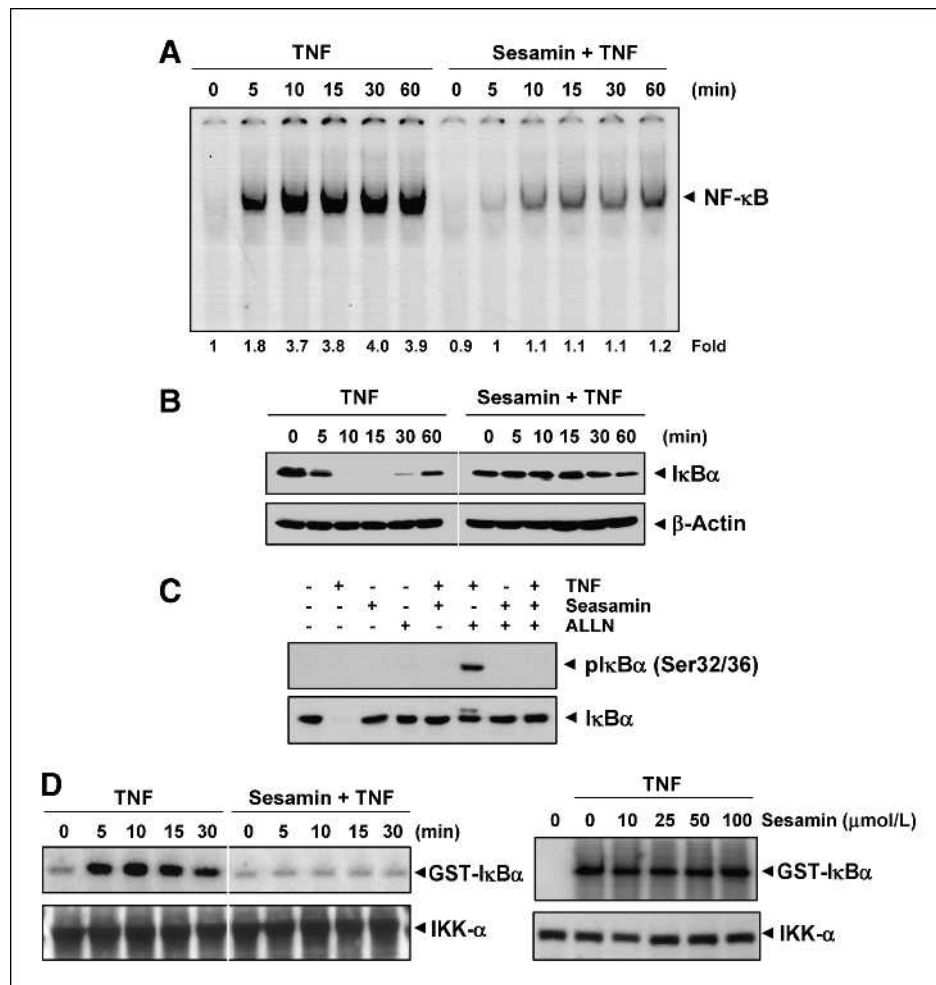


FIGURE 4. A, sesamin inhibits TNF-induced activation of NF- κ B. KBM-5 cells were incubated with 100 μ mol/L sesamin for 12 h, treated with 0.1 nmol/L TNF for the indicated time intervals, and then analyzed by EMSA for NF- κ B activation. B, effect of sesamin on TNF-induced degradation of I κ B α . KBM-5 cells were incubated with 100 μ mol/L sesamin for 12 h and treated with 0.1 nmol/L TNF for the indicated times. Cytoplasmic extracts were prepared and analyzed by Western blotting using antibodies against anti-I κ B α . Equal protein loading was evaluated by β -actin. C, effect of sesamin on phosphorylation of I κ B α induced by TNF. Cells were preincubated with 100 μ mol/L sesamin for 12 h, incubated with 50 μ g/mL *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) for 30 min, and then treated with 0.1 nmol/L TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phospho-specific I κ B α antibody. D, effect of sesamin on the activation of IKK by TNF. Left, KBM-5 cells were preincubated with 100 μ mol/L sesamin for 12 h and then treated with 1 nmol/L TNF for the indicated time points. Whole-cell extracts were immunoprecipitated with antibody against IKK- α and analyzed by an immune complex kinase assay. To examine the effect of sesamin 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 antibody. Right, direct effect of sesamin on IKK activation induced by TNF. Whole-cell extracts were prepared from KBM-5 cells treated with 1 nmol/L TNF and immunoprecipitated with anti-IKK- α antibody. The immunocomplex kinase assay was done in the absence or presence of the indicated concentration of sesamin.

the effect of sesamin on TNF-induced phosphorylation of p65. We found that in a time-dependent manner, phosphorylation of p65 occurred in TNF-treated KBM-5 cells. We also observed that in sesamin pretreated cells, there was no phosphorylation of p65 (Fig. 5B).

Sesamin suppressed TNF-induced NF- κ B-dependent reporter gene expression. Although we showed using EMSA that sesamin inhibited TNF-induced NF- κ B expression, DNA binding alone does not always correlate with NF- κ B-dependent gene transcription, suggesting that additional regulatory steps are involved. Thus, we decided to examine whether sesamin affects TNF-induced reporter gene transcription. For this, cells were transiently transfected with a NF- κ B-regulated SEAP reporter construct, followed by treatment with sesamin, and then exposed to TNF. We found that TNF induced NF- κ B reporter gene activity, and in a dose-dependent manner, sesamin inhibited the TNF-induced NF- κ B reporter activity (Fig. 6A).

Sesamin inhibits NF- κ B activation induced by TNFR1, TRADD, NIK, TAK1/TAB1, and IKK. TNF-induced NF- κ B activation requires a sequential recruitment of TNFR1, TRADD, TAK1, and IKK (30, 31). To determine where in the pathway sesamin blocks the TNF-induced NF- κ B activation, we decided to examine the effect of sesamin on TNFR1, TRADD, NIK, TAK1/TAB1, and IKK-induced NF- κ B-dependent reporter gene transcription. We observed that all of these plasmids induced NF- κ B reporter activity and sesamin was found to inhibit the activation (Fig. 6B).

Discussion

Although numerous studies have indicated that sesamin exhibits activity against hypertension, hyperlipidemia, septic shock, and carcinogenesis, its precise mechanism of action is not understood. Antihypertensive effects have been documented even in human clinical trials (32). Because inflammation has been linked with most chronic diseases including cancer, it is possible that modulation of inflammatory pathway is one of the major sites of action of sesamin. Over the last decade, NF- κ B pathway has emerged as a major mediator of inflammation (19, 20). The major aim of the current study was to determine the effects of sesamin on NF- κ B-mediated cellular responses linked to the prevention of cancer. We found that sesamin inhibited the NF- κ B pathway induced by various carcinogens, inflammatory stimuli, and cytokines. It also inhibited constitutive expression of NF- κ B activation.

We found sesamin suppressed the proliferation of a wide variety of tumor cells including leukemia and solid tumor cells of the prostate, colon, pancreas, lung, and breast. Suppression of proliferation of these cells is most likely linked to the inhibition of gene products linked with the survival and proliferation of cells such as Bcl-2, survivin, cyclin D1, and COX-2.

By using DNA binding assay, we showed NF- κ B activated by highly diverse stimuli was blocked by sesamin, suggesting that sesamin acts at a step common to all. Our results agree with a recent report about suppression of LPS-induced NF- κ B monitored by nuclear pool of p65

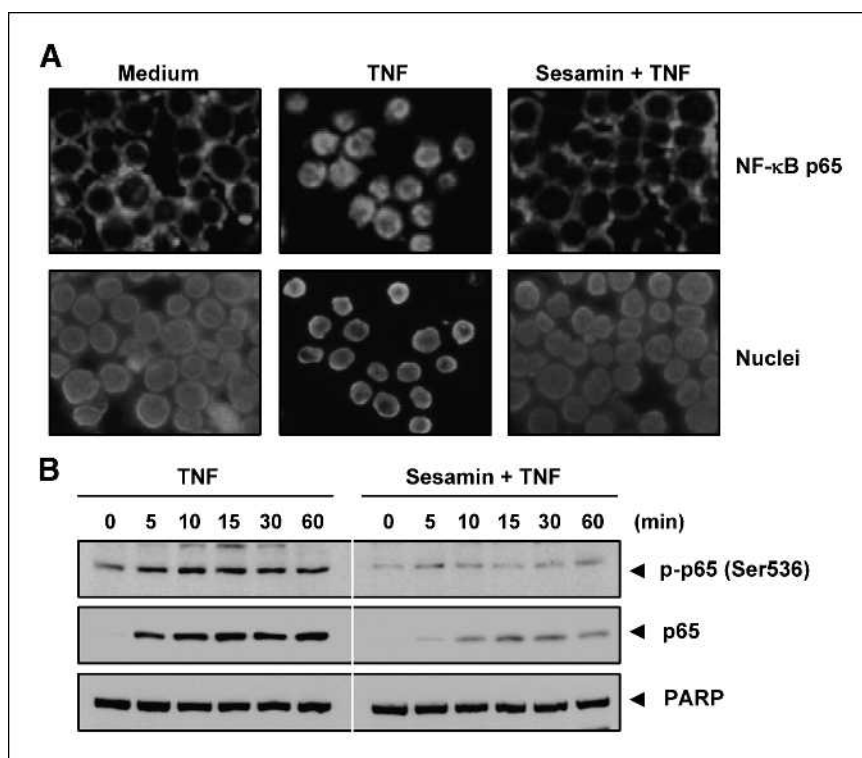
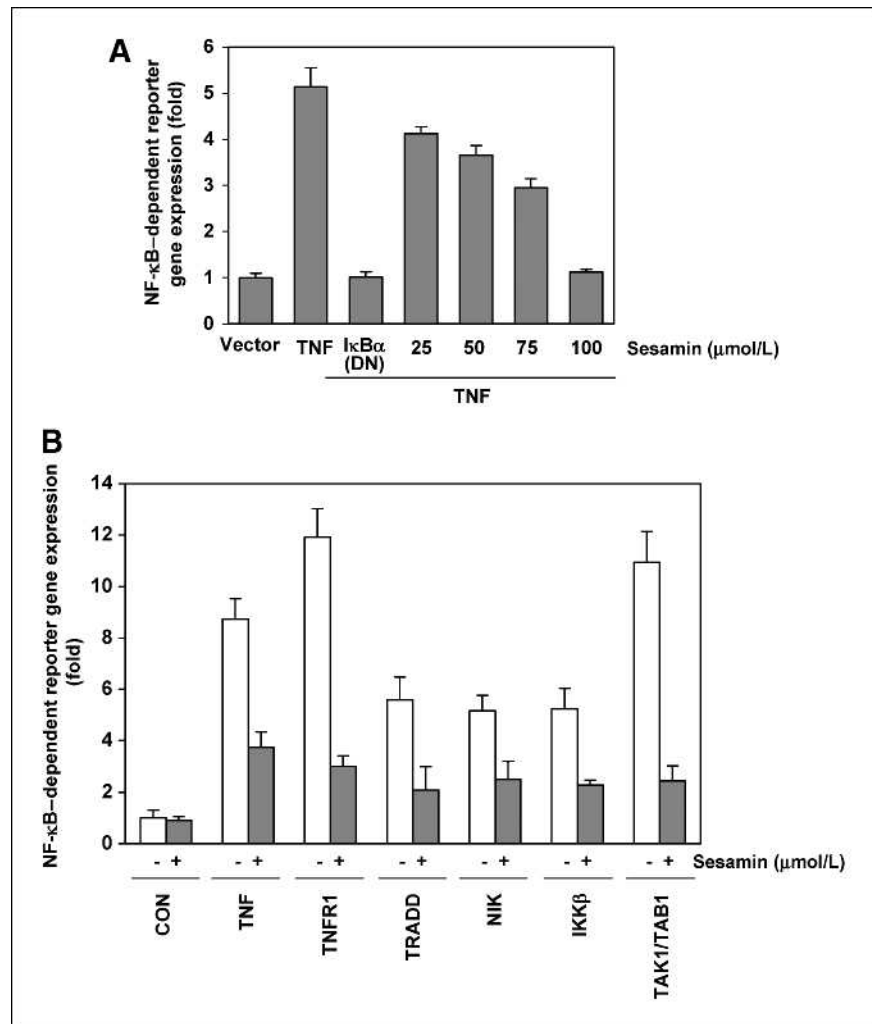


FIGURE 5. A, sesamin inhibits the TNF-induced nuclear translocation of p65 assayed by immunocytochemical analysis. KBM-5 cells were initially treated with 100 μ mol/L sesamin for 12 h and then exposed to 0.1 nmol/L TNF for 15 min. After cytospin, immunocytochemical analysis was done as described under Materials and Methods. B, sesamin inhibits the TNF-induced phosphorylation of p65. KBM-5 cells were either nontreated or pretreated with 100 μ mol/L sesamin for 12 h and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using p65 and phospho-specific p65 antibodies. For loading control of nuclear extracts, the membrane was reblotted with anti-poly ADP ribose polymerase (PARP) antibody, respectively.

FIGURE 6. A, sesamin inhibits TNF-induced NF- κ B-dependent reporter gene (*SEAP*) expression. A293 cells treated were transiently transfected with a NF- κ B-containing plasmid linked to the *SEAP* gene. After 24 h of transfection, cells were treated with the indicated concentrations of sesamin for 12 h and with 1 nmol/L TNF for 24 h; cell supernatants were collected and assayed for *SEAP* activity as described under Materials and Methods. Results are expressed as fold activity over the activity of the vector control. B, sesamin inhibited NF- κ B-dependent reporter gene expression induced by TNF, TNFR1, TRADD, NIK, IKK, and TAK1/TAB1. A293 cells were transiently transfected with the indicated plasmids along with a NF- κ B-containing plasmid linked to the *SEAP* gene, and after 24 h, cells were either nontreated or treated with 100 μ mol/L sesamin for 12 h. Where indicated, cells were exposed to 1 nmol/L TNF for 24 h. Cell supernatants were assayed for *SEAP* activity as described under Materials and Methods. Results are expressed as fold activity over the activity of the vector control. DN, dominant negative.



in microglia cells (17). How sesamin inhibits LPS-induced NF- κ B activation was not examined by these investigators. We found that sesamin inhibited the activation of IKK, thereby preventing the phosphorylation as well as degradation of I κ B α . When we examined the effects of sesamin on IKK in detail, we found that this lignan did not directly modulate the activity of IKK. However, it blocked the activation of the kinase. Numerous kinases have been linked with the activation of IKK. TAK1 is one of the kinase that has been shown to mediate TNF-induced NF- κ B (31). We found that sesamin blocked TNF-induced TAK1-mediated NF- κ B activation. IKK has also been shown to mediate the phosphorylation of p65, the DNA binding subunit (28). We found that sesamin also inhibited the phosphorylation of p65. NF- κ B reporter gene expression induced by TNF and TNF signaling components was also suppressed by this lignan.

When examined for the expression of antiapoptotic gene products, survivin and Bcl-2, both regulated by NF- κ B, were suppressed by sesamin. In addition, sesamin inhibited the expression of protein COX-2 closely linked with inflammation. Although sesamin has been shown to exhibit

anti-inflammatory activity and downregulate prostaglandin production (7, 8, 10), ours is the first report to show that this agent can downregulate COX-2 expression. Reports about the anti-inflammatory activity due to the downregulation of IL-1, IL-6 (10), nitric oxide (16), and thromboxane B2 could also be due to its ability to downregulate NF- κ B as described here, as inducible nitric oxide synthase and lipooxygenase are also regulated by NF- κ B. Lee et al. (33) showed that sesamin inhibited the expression of phospholipase C- γ 1 but the mechanism was not shown. Because phospholipase C- γ 1 expression is also regulated by NF- κ B (34), downregulation of NF- κ B signaling by sesamin may decrease the expression of phospholipase C- γ .

Our results also indicate that sesamin inhibition of NF- κ B led to the downregulation of the expression cyclin D1 closely associated with proliferation of cells. This agrees with the observations of Yokota et al. (18). It is possible that reports that indicated the suppression of proliferation of various tumor cells, including leukemia (12, 13), breast cancer (18), and gastric cancer (14), is due to the suppression of cyclin D1 expression.

We also found for the first time that the expression of protein linked with adhesion (ICAM-1), invasion (MMP-9), and angiogenesis (VEGF) was also abrogated by sesamin. Although sesamin has been shown to inhibit 7,12-dimethylbenz[a]anthracene-induced breast carcinogenesis in rats, it is possible that these effects of sesamin involve suppression of the NF- κ B pathway. 7,12-Dimethylbenz[a]anthracene indeed has been shown to activate NF- κ B (35).

Additionally, we also found that the downregulation of expression of antiapoptotic gene products led to the enhancements of apoptosis induced by cytokines as well as by chemotherapeutic agents. That NF- κ B activation can inhibit apoptosis and induce chemoresistance is well established (19, 36). These studies suggest that sesamin can be used not only alone but also in combination with existing therapies both to potentiate their effect and to overcome chemoresistance.

One of the earliest activity reported of sesamin is its ability to lower cholesterol (3). Like sesamin, well-known cholesterol-lowering drugs such as statin have also been shown to suppress the NF- κ B pathway and sensitize the cells to chemotherapeutic agents (37, 38). Besides numerous ani-

mal studies (39), several clinical trials have been done with sesamin, showing its safety and bioavailability (1, 32, 40-42). In conclusion, our results show the mechanism by which sesamin could mediate anti-inflammatory, antiproliferative, and antiangiogenic effects against cancer through the inhibition of NF- κ B and NF- κ B-regulated gene products. A further investigation in animals and humans with sesamin is needed to fully realize its potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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