Oncogene (2005), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Diosgenin inhibits osteoclastogenesis, invasion, and proliferation through the downregulation of Akt, IκB kinase activation and NF-κB-regulated gene expression

S Shishodia and BB Aggarwal

Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Diosgenin, a steroidal saponin present in fenugreek (*Trigonella foenum graecum*) and other plants, has been shown to suppress inflammation, inhibit proliferation, and induce apoptosis in a variety of tumor cells, but through a mechanism that is poorly understood. In the present study, we report that diosgenin inhibits receptor-activated nuclear factor-kappaB ligand-induced osteoclastogenesis, suppresses tumor necrosis factor (TNF)-induced invasion, and blocks the proliferation of tumor cells, all activities known to be regulated by NF-κB. Diosgenin suppressed TNF-induced NF-κB activation as determined by DNA binding, activation of IκBα kinase, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation through inhibition of Akt activation. NF-κB-dependent reporter gene expression was also abrogated by diosgenin. TNF-induced expression of NF-κB-regulated gene products involved in cell proliferation (cyclin D1, COX-2, c-myc), antiapoptosis (IAP1, Bcl-2, Bcl-X_L, Bfl-1/A1, TRAF1 and cFLIP), and invasion (MMP-9) were also downregulated by the saponin. Diosgenin also potentiated the apoptosis induced by TNF and chemotherapeutic agents. Overall, our results suggest that diosgenin suppresses proliferation, inhibits invasion, and suppresses osteoclastogenesis through inhibition of NF-κB-regulated gene expression and enhances apoptosis induced by cytokines and chemotherapeutic agents.

Oncogene advance online publication, 5 December 2005; doi:10.1038/sj.onc.1209194

Keywords: NF-κB; IKK; Akt; invasion; osteoclastogenesis; diosgenin

Correspondence: Professor BB Aggarwal, Cytokine Research Laboratory, Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Box 143,1515 Holcombe Boulevard, Houston, TX 77030, USA.
E-mail: aggarwal@mdanderson.org
Received 2 July 2005; revised 12 September 2005; accepted 12 September 2005

Introduction

Although it is generally believed that traditional medicine has great value, the molecular basis for their activities is for the most part lacking (Djerassi, 1992). One of the traditional medicines whose mechanisms are largely unknown, diosgenin, is a steroidal saponin found in a variety of plants (Puri *et al*., 1976; Djerassi, 1992) including fenugreek (*Trigonella foenum graecum*), roots of wild yam (*Dioscorea villosa*), *Solanum incanum* Lloydia (Segal *et al*., 1977), *Costus speciosus* (Dasgupta and Pandey, 1970), and *Solanum xanthocarpum* (Heble *et al*., 1967). Extracts from these plants have been traditionally used for the treatment of diabetes (Madar *et al*., 1988; Sharma *et al*., 1990; Gupta *et al*., 2001), hypercholestrolemia (Valette *et al*., 1984; Sauvaire *et al*., 1991), and gastrointestinal ailments (Pandian *et al*., 2002; Raju *et al*., 2004). Research during the last decade has shown that diosgenin suppresses proliferation and induces apoptosis in cells of human colon carcinoma (Raju *et al*., 2004; Wang *et al*., 2004), osteosarcoma (Moalic *et al*., 2001; Corbiere *et al*., 2003), leukemia (Hibasami *et al*., 2003; Liu *et al*., 2005), human erythroleukemia (Leger *et al*., 2004), and human rheumatoid arthritis (Liagre *et al*., 2004). Antiproliferative effects of diosgenin are mediated through cell cycle arrest (Moalic *et al*., 2001; Liu *et al*., 2005), disruption of Ca^{2+} homeostasis (Leger *et al*., 2004; Liu *et al*., 2005), the activation of p53, release of apoptosis-inducing factor, and modulation of caspase-3 activity (Corbiere *et al*., 2004). It also inhibits azoxymethane-induced aberrant colon crypt foci (Raju *et al*., 2004) and has been shown to inhibit intestinal inflammation (Yamada *et al*., 1997) and modulate the activity of lipoxygenase (LOX) (Nappez *et al*., 1995) and cyclooxygenase-2 (COX-2) (Moalic *et al*., 2001). More recently, diosgenin has been shown to bind to the chemokine receptor CXCR3, which mediates inflammatory responses (Ondeykal *et al*., 2005).

Since the transcription factor NF-κB is a major mediator of inflammation, cell survival, LOX, and COX-2 expression, we postulated that the anti-inflammatory and antiapoptotic effects of diosgenin are modulated through the suppression of NF-κB. NF-κB is a family of five proteins, namely c-Rel, RelA (p65),

Rel B, NF-κB1 (p50 and p105) and NF-κB2 (p52) (Aggarwal, 2004) that is kept in an inactive state in the cytoplasm by the members of the inhibitory subunit of NF-κB (IκB) family, which includes IκBα, IκBβ, IκBε, IκBγ, Bcl-3, p100, and p105 (Aggarwal, 2004). The most common combination is a heterotrimer consisting of p50, p65, and IκBα subunits. In response to an activation signal, the IκBα subunit is phosphorylated, ubiquitinated, and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50–p65 heterodimer. The p65 is then phosphorylated, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. NF-κB has been shown to regulate the expression of a number of genes whose products are involved in tumorigenesis (Aggarwal, 2004; Shishodia and Aggarwal, 2004b). These include antiapoptosis genes (e.g. *ciap, suvivin, traf, bcl-2, and bcl-xl*); *cox-2*; matrix metalloproteinase-9 (*mmp-9*); genes encoding adhesion molecules, chemokines, inflammatory cytokines, and *inos*; and cell cycle regulatory genes (e.g. *cyclin d1*). NF-κB has also been shown to mediate receptor-activated nuclear factor-kappaB (NF-κB) ligand (RANKL)-induced osteoclastogenesis (Aggarwal, 2003).

The aim of the current study was to investigate the effect of diosgenin on tumor necrosis factor (TNF)-induced invasion, RANKL-induced osteoclastogenesis, NF-κB activation, and NF-κB-regulated gene products. We found that diosgenin inhibited TNF-induced invasion of tumor cells and osteoclastogenesis induced by RANKL through the inhibition of NF-κB and NF-κB-regulated gene products. Diosgenin also potentiated the apoptosis induced by TNF and the chemotherapeutic drugs doxorubicin and Taxol.

Results

The aim of this study was to investigate the effect of the plant steroid diosgenin on the transcription factor NF-κB-signaling pathway and on NF-κB-regulated gene products. The structure of this compound is shown in Figure 1a. For most studies, human chronic myelogenous leukemia (KBM-5) cells were used. The concentration of diosgenin used and the duration of exposure had minimal effect on the viability of KBM-5 cells as determined by trypan blue dye exclusion test (data not shown). We used TNF to examine the effect of diosgenin on the NF-κB activation pathway because the pathway activated by this agent is well understood.

Diosgenin suppresses RANKL-induced osteoclastogenesis
RANKL, a member of the TNF superfamily, induces osteoclastogenesis through the activation of NF-κB (Abu-Amer and Tondravi, 1997), and inhibits the apoptosis of osteoclasts, which subsequently leads to bone loss. We first determined whether diosgenin could suppress RANKL-induced osteoclastogenesis. We found that RANKL induced osteoclast differentiation in RAW macrophage cell lines, as indicated by the expression of TRAP, and that diosgenin suppressed it (Figure 1b). RANKL induced 250 osteoclasts per well, and diosgenin suppressed induction to 80 osteoclasts per well (Figure 1c).

Diosgenin suppresses TNF-induced invasion activity
TNF can induce tumor metastasis-related genes such as *mmp-9, cox-2, and icam-1* (Aggarwal, 2003). Tumor metastasis depends on the activity of MMPs, COX-2, and adhesion molecules (Liotta *et al*., 1982). Thus, we investigated whether diosgenin can modulate the tumor cell invasion activity induced by TNF *in vitro*. H1299 cells were seeded in the top chamber of the Matrigel invasion chamber in the absence of serum, incubated with diosgenin, and then treated with TNF in the presence of 1% serum with diosgenin. Diosgenin suppressed TNF-induced invasion activity by 50% (Figure 1d). We also examined the effect of diosgenin post-treatment on TNF-induced invasive activity. We observed that post-treatment with diosgenin inhibited TNF induced invasive activity by over 40% (Figure 1e).

Diosgenin suppresses proliferation, and induces apoptosis
We next examined the effect of diosgenin on proliferation of KBM-5 cells by the MTT method (Figure 2a) and [^{3}H]thymidine incorporation (Figure 2b). Diosgenin at a concentration of 25 μM inhibited growth and proliferation of KBM-5 cells. Next, we examined the effect of diosgenin on the cell cycle. Flow cytometric analysis of the DNA from diosgenin-treated cells showed a significant increase in the percentage of cells in the sub-G1 phase, from 9 to 51%, within 48 h of diosgenin (50 μM) treatment (Figure 2c). An accumulation of cells in the sub-G1 phase indicates that diosgenin induced the apoptosis of cells.

Diosgenin suppresses the activation of NF-κB in a dose- and time-dependent manner
Our results showed that diosgenin inhibited TNF-induced invasion, RANKL-induced osteoclastogenesis and expression of cyclin D1 and suppressed proliferation in tumor cells. Since NF-κB has been implicated in osteoclastogenesis, invasion, cell survival, and proliferation (Aggarwal, 2004; Shishodia and Aggarwal, 2004b), that are all regulated by NF-κB, we examined the effect of diosgenin on TNF-induced NF-κB activation. Diosgenin inhibited TNF-mediated NF-κB activation in a dose-dependent manner (Figure 3a) with significant inhibition occurring at 50 μM and complete abrogation of NF-κB activity occurring at 100 μM. Diosgenin by itself did not activate NF-κB. Then, we examined the time kinetics of NF-κB inhibition by diosgenin. The minimum time required for inhibition of NF-κB activation with 50 μM diosgenin was 24 h (Figure 3b).

Suppressed NF-κB consists of both p50 and p65
Since NF-κB is a family of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in

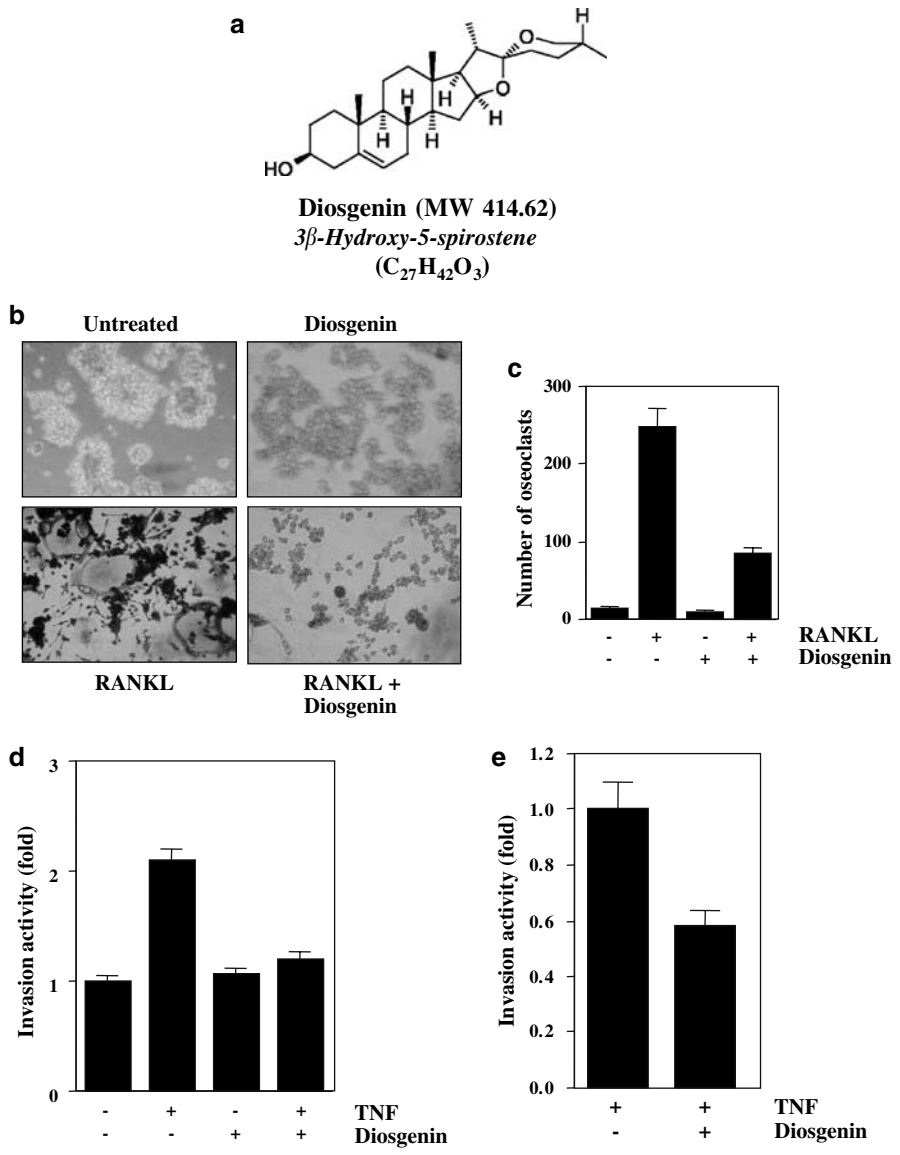

Figure 1 (**a**) Structure of diosgenin. (**b**) Diosgenin suppresses TNF-induced osteoclastogenesis. RAW 264.7 cells (1×10^4) were plated overnight, pretreated with 5 μM diosgenin for 12 h, and then treated with 5 nM RANKL. After 5 days, cells were stained for TRAP and evaluated for osteoclastogenesis. Photographs were taken after 5 days incubation with RANKL. (**c**) The number of TRAP-positive multinucleated osteoclasts (> 3 nuclei) per well were counted. (**d**) Diosgenin pretreatment suppresses TNF-induced invasive activity. H1299 cells (2.5×10^4) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 10 μM diosgenin for 12 h, treated with 1 nM TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay. The value for no diosgenin and no TNF was set to 1. (**e**) Diosgenin suppresses TNF-induced invasive activity. H1299 cells (2.5×10^4) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, treated with TNF (1 nM) alone or in combination with diosgenin (10 μM) for 24 h and then subjected to invasion assay. The value for TNF alone was set to 1.

DNA (Ghosh *et al*., 1998). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed the p50 and p65 subunits of NF-κB, we incubated nuclear extracts from TNF-activated cells with antibodies to the p50 (NF-κB1) and the p65 (RelA) subunit of NF-κB. Both antibodies shifted the band to a higher molecular mass (Figure 3c), suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum had no effect on DNA binding. Addition of excess unlabeled NF-κB (cold oligo; 100-fold) caused complete disappearance of the band, whereas mutated oligo had no effect on the DNA binding.

Diosgenin represses TNF-induced NF-κB-dependent reporter gene expression

Although we showed by EMSA that diosgenin blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting there are additional regulatory steps (Nasuhara *et al*., 1999). To further determine whether

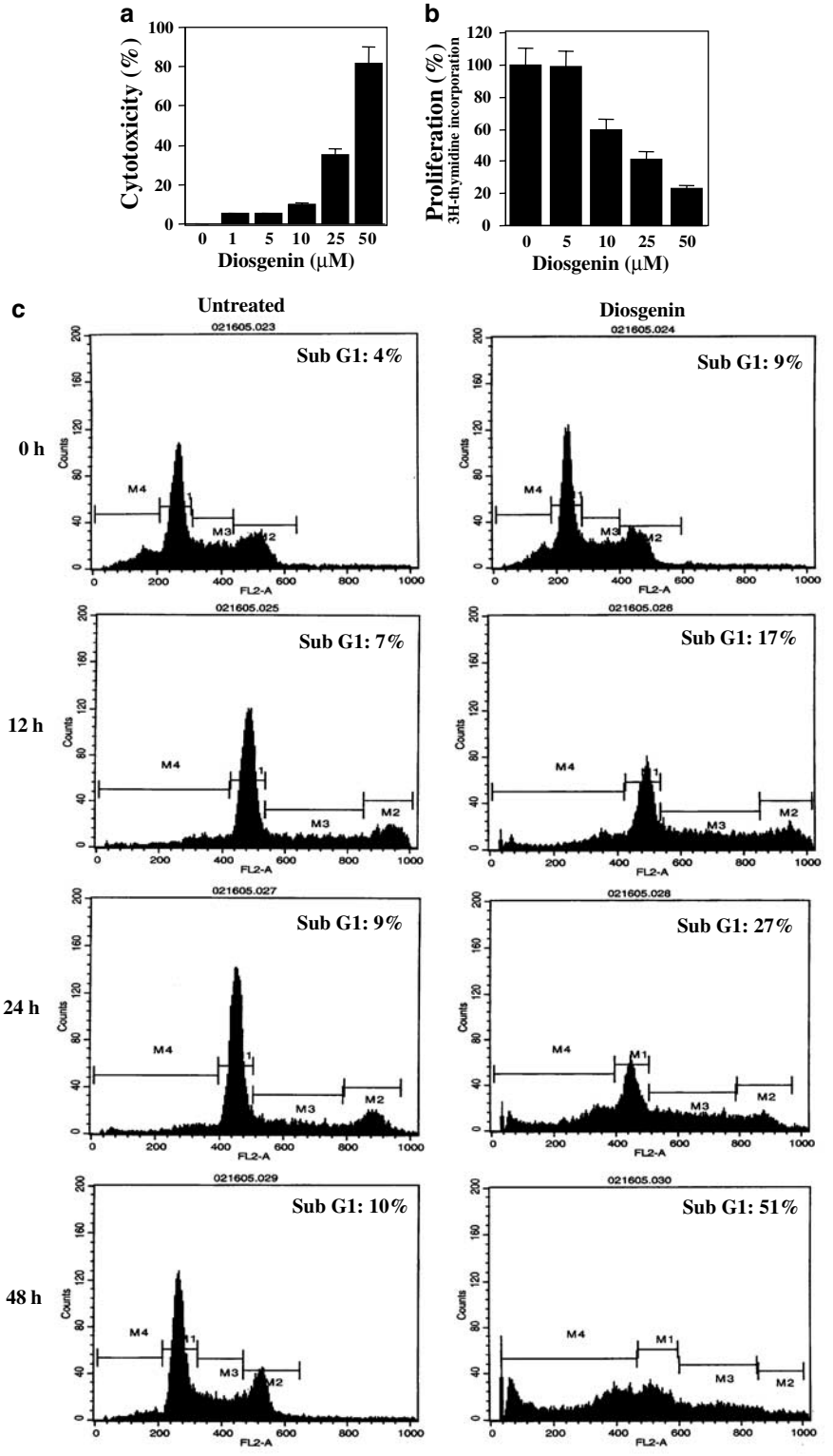

Figure 2 Diosgenin induces cytotoxicity and inhibits proliferation in KBM-5 cells. (**a**) KBM-5 (5000 cells/0.1 ml) were incubated at 37°C with indicated concentrations of diosgenin for 72 h, and the viable cells were assayed using MTT reagent. The results are shown as the mean ± s.d. from triplicate cultures. (**b**) KBM-5 (5000 cells/0.1 ml) were incubated at 37°C with indicated concentrations of diosgenin for 72 h, and the viable cells were assayed using [^{3}H]thymidine incorporation as described in Materials and methods. The results are shown as the mean ± s.d. from triplicate cultures. (**c**) Diosgenin accumulates the cells at the sub-G1 phase of the cell cycle. Serum-starved KBM-5 cells (2×10^6 cells/ml) were incubated in the absence or in presence of 25 μM diosgenin for indicated times. Thereafter, the cells were washed, fixed, stained with propidium iodide, and analysed for DNA content by flow cytometry as described in Materials and methods.

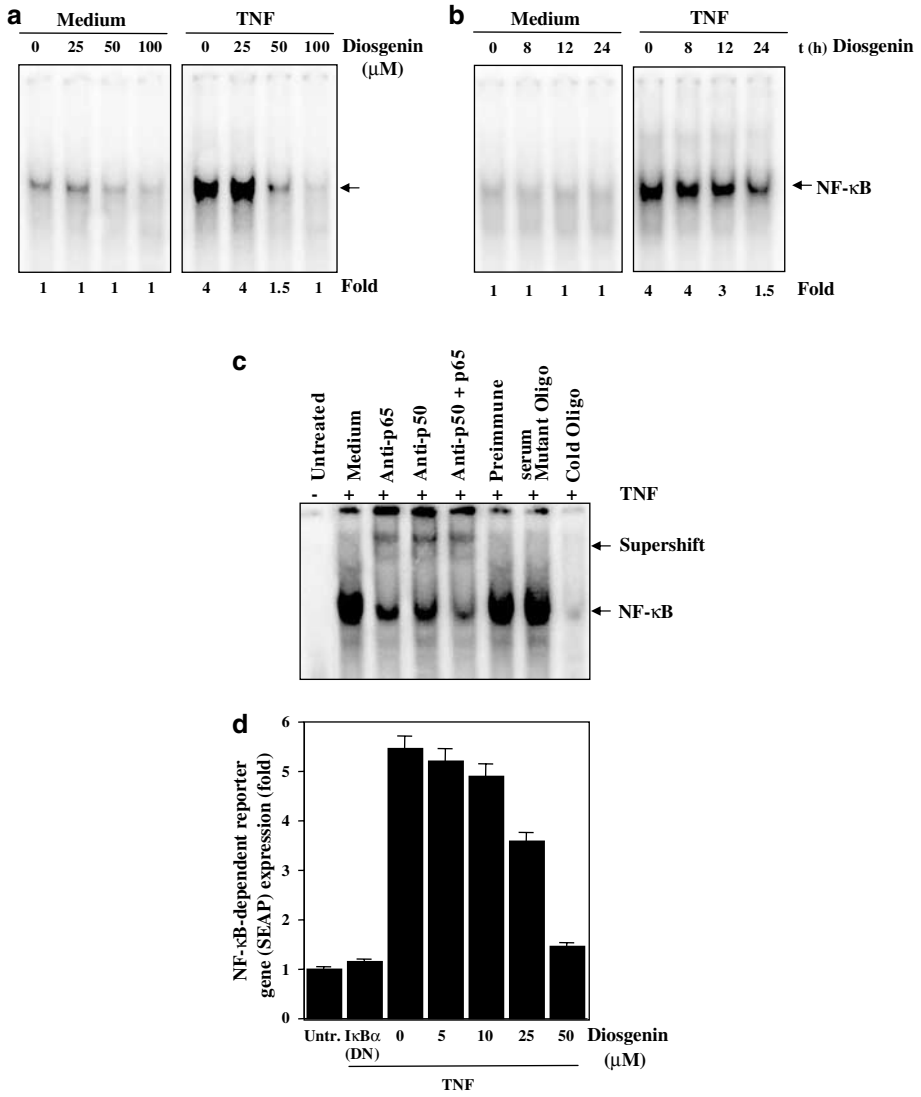

Figure 3 Diosgenin inhibits NF-κB. (**a**) Diosgenin inhibits TNF-dependent NF-κB activation in a dose-dependent manner. KBM-5 cells (2×10^6/ml) were preincubated with the indicated concentrations of diosgenin for 24 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation, as described in Materials and methods. (**b**) Diosgenin inhibits TNF-dependent NF-κB activation in a time-dependent manner. KBM-5 cells (2×10^6/ml) were preincubated with 50 μM diosgenin for the indicated times at 37°C and then treated with 0.1 nM TNF for 30 min at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. (**c**) TNF-induced NF-κB consists of p50 and p65 subunits. Nuclear extracts from KBM-5 cells (2×10^6/ml) treated or not treated with 0.1 nM TNF for 30 min were incubated with the antibodies indicated for 30 min at room temperature, and the complex was analysed by supershift assay. (**d**) Diosgenin inhibits TNF-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with an NF-κB-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of diosgenin. After 24 h in culture with 0.1 nM TNF, cell supernatants were collected and assayed for SEAP activity as described in Materials and methods. Results are expressed as fold activity over the activity of the vector control.

diosgenin inhibited NF-κB-dependent gene transcription, we transiently transfected A293 cells with the NF-κB-regulated secretory alkaline phosphatase (SEAP) reporter construct and then stimulated them with TNF. We found that TNF produced an almost fivefold increase in SEAP activity over vector control (Figure 3d), which was inhibited by dominant-negative IκBα, indicating specificity. When the cells were pretreated with diosgenin, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results demonstrate that diosgenin inhibits NF-κB-dependent reporter gene expression induced by TNF.

Diosgenin inhibits TNF-dependent IκBα degradation

Since IκBα degradation is required for translocation of NF-κB to the nucleus (Miyamoto *et al*., 1994), we determined whether diosgenins' inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation. We pretreated cells with diosgenin and then exposed them to TNF for different times. We

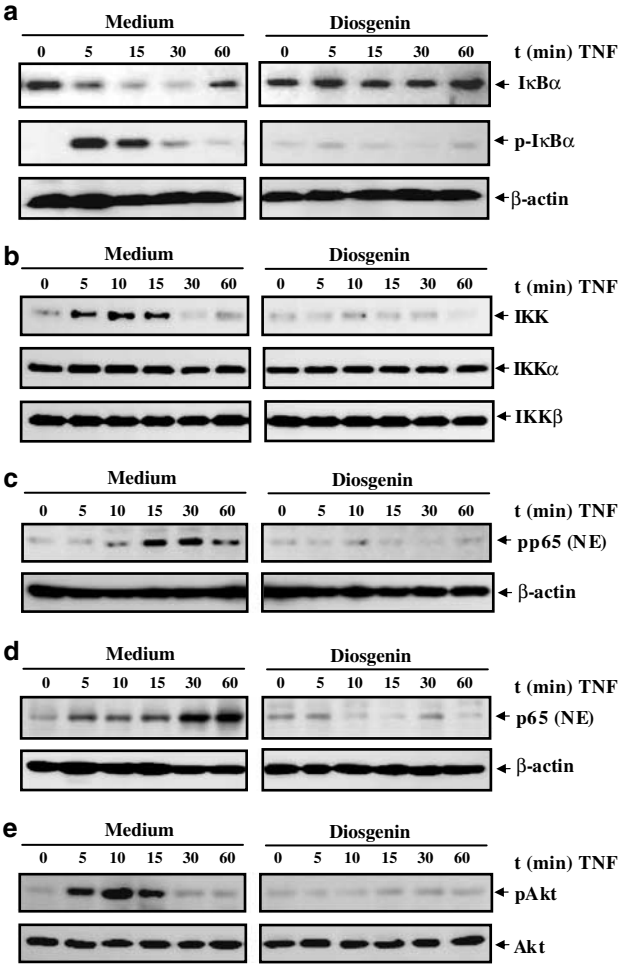

Figure 4 (**a**) Diosgenin inhibits TNF-induced degradation and phosphorylation of IκBα. KBM-5 cells (2×10^6/ml) were incubated with 50 μM diosgenin for 24 h at 37°C, treated with 0.1 nM TNF for the indicated times at 37°C, and then tested for IκBα (upper panel) and phosphorylated IκBα (middle panel) in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (lower panel). (**b**) Diosgenin inhibits TNF-induced IKK activity. KBM-5 cells (2×10^6/ml) were treated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for the indicated time intervals. Whole-cell extracts were prepared, and 200 μg of extract was immunoprecipitated with antibodies against IKKα and IKKβ. Thereafter immune complex kinase assay was performed as described in Materials and methods. To examine the effect of diosgenin on the level of expression of IKK proteins, 30 μg of whole-cell extract was run on 10% SDS–PAGE, electrotransferred, and immunoblotted with indicated antibodies as described in Materials and methods. (**c**) Diosgenin inhibits TNF-induced phosphorylation of p65. KBM-5 cells (2×10^6/ml) were incubated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for the indicated times. The cytoplasmic extracts were analysed by Western blotting using antibodies against the phosphorylated form of p65. (**d**) Diosgenin inhibits TNF-induced nuclear translocation of p65. KBM-5 cells (1×10^6/ml) were either untreated or pretreated with 50 μM diosgenin for 24 h at 37°C and then treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and analysed by Western blotting using antibodies against p65. (**e**) Diosgenin inhibits Akt phosphorylation. KBM-5 cells (2×10^6/ml) were incubated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for the indicated times. The whole-cell extracts were analysed by Western blotting using antibodies against the phosphorylated Akt. Equal protein loading was evaluated by Akt. Data are from a representative experiment out of the three independent ones showing similar results.

then examined the cells for IκBα in the cytoplasm by Western blot analysis. We found that TNF induced IκBα degradation in control cells as early as 15 min, but in diosgenin-pretreated cells TNF had no effect on IκBα degradation (Figure 4a, upper panel).

Diosgenin inhibits TNF-dependent IκBα phosphorylation

We next determined whether diosgenin affected TNF-induced IκBα phosphorylation, another condition for NF-κB translocation (Aggarwal, 2004). Western blot analysis using antibody that detects only the serine-phosphorylated form of IκBα indicated that diosgenin completely suppressed TNF-induced IκBα phosphorylation as early as 5 min, and diosgenin completely suppressed it (Figure 4a, middle panel). Thus diosgenin inhibited TNF-induced NF-κB activation by inhibiting phosphorylation and degradation of IκBα.

Diosgenin inhibits TNF-induced IκBα kinase (IKK) activation

The phosphorylation of IκBα is catalysed by the IKK. IKK consists of three subunits IKK-α, IKK-β and IKK-γ (also called NEMO). Gene deletion studies have indicated that IKK-β is essential for NF-κB activation by most agents (Aggarwal, 2004). Since diosgenin inhibits the phosphorylation and degradation of IκBα, we tested the effect of diosgenin on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of IκBα (Aggarwal, 2004). As shown in Figure 4b (upper panel), diosgenin completely suppressed TNF-induced activation of IKK. TNF or diosgenin had no direct effect on the expression of IKK proteins (bottom panels).

Diosgenin inhibits TNF-induced phosphorylation and nuclear translocation of p65

We also tested the effect of diosgenin on TNF-induced phosphorylation of p65, since phosphorylation is also required for transcriptional activity of p65 (Zhong *et al*., 1998). As shown in Figure 4c, diosgenin suppressed p65 phosphorylation almost completely. Likewise, Western blot analysis (Figure 4d) indicated that diosgenin abolished TNF-induced nuclear translocation of p65.

Diosgenin inhibits TNF-induced Akt activation

TNF has been shown to activate IKK through activation of Akt (Ozes *et al*., 1999). Therefore, we also tested the effect of diosgenin on TNF-induced activation of Akt. As shown in Figure 4e, diosgenin treatment suppressed TNF-induced activation of Akt.

Diosgenin inhibits TNF-induced activation of antiapoptotic gene products

NF-κB upregulates the expression of a number of genes implicated in facilitating tumor cell survival, including BCl-2, BclXL, cIAP1, cFLIP, and Bfl-1 (Aggarwal, 2004). We found that diosgenin inhibited both the basal and the TNF-induced expression of all of these gene products (Figure 5a). Treatment of cells with 50 μM

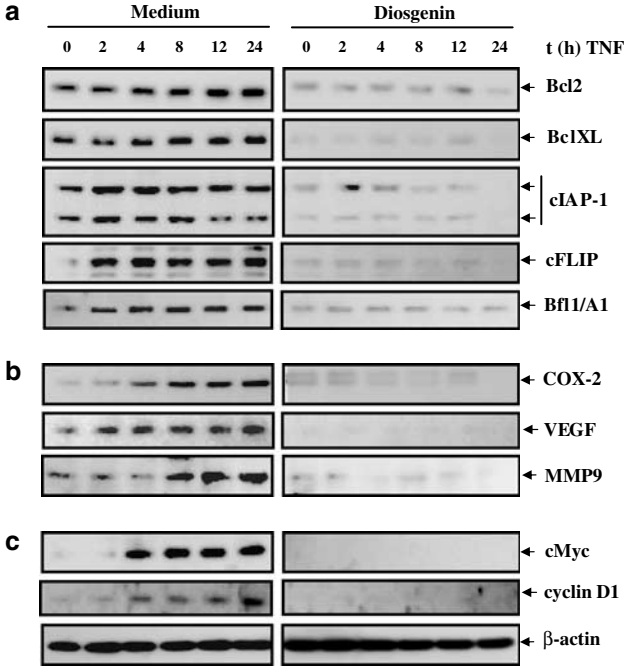

Figure 5 Diosgenin inhibits TNF-induced NF-κB-regulated gene products. (**a**) Diosgenin inhibits the expression of antiapoptotic gene products Bcl-2, BclXL, cIAP1, XIAP-1, cFLIP, and Bfl-1/A1: KBM-5 cells (2×10^6/ml) were left untreated or incubated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for different times. Whole-cell extracts were prepared, and 50 μg of the whole-cell lysate was analysed by Western blotting using antibodies as indicated. Data are from a representative experiment out of the three independent ones showing similar results. (**b**) Diosgenin inhibits COX-2, VEGF, and MMP-9 expression induced by TNF. KBM-5 cells (2×10^6/ml) were left untreated or incubated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for different times. Whole-cell extracts were prepared, and 80 μg of the whole-cell lysate was analysed by Western blotting using antibodies against COX-2, VEGF, and MMP-9. (**c**) Diosgenin inhibits c-Myc and cyclin D1 expression induced by TNF. KBM-5 cells (2×10^6/ml) were left untreated or incubated with 50 μM diosgenin for 24 h and then treated with 0.1 nM TNF for different times. Whole-cell extracts were prepared, and 80 μg of the whole-cell lysate was analysed by Western blotting using antibodies against c-myc and cyclin D1. Data are from a representative experiment out of the three independent ones showing similar results.

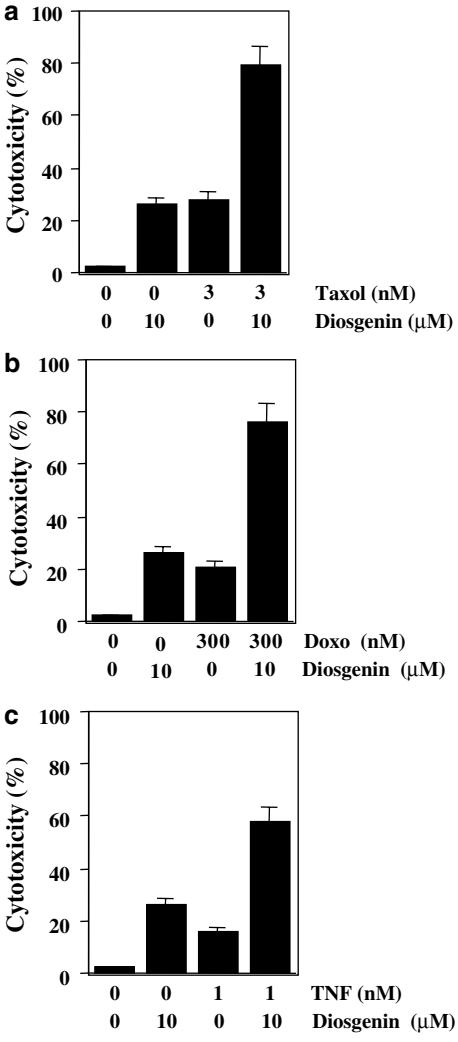

Figure 6 Diosgenin enhances apoptosis induced by TNF and chemotherapeutic agents. KBM-5 cells (5000 cells/0.1 ml) were incubated at 37°C with Taxol, TNF, or doxorubicin (Doxo) in the presence and absence of 10 μM diosgenin, as indicated, for 72 h duration, and the viable cells were assayed using MTT reagent. The results are expressed as mean cytotoxicity ± s.d. from triplicate cultures.

diosgenin for 24 h had cell viability greater than 85% (data not shown).

Diosgenin inhibits TNF-induced COX-2, MMP-9, and VEGF expression

Diosgenin abolished, in a time-dependent fashion, both basal and TNF induced expression of VEGF, COX-2, and MMP-9 gene products (Figure 5b), which are known to be NF-κB-regulated gene products (Aggarwal, 2004).

Diosgenin inhibits TNF-induced cyclin D1 and c-myc expression

c-myc and cyclin D1 regulates cellular proliferation and is regulated by NF-κB (Aggarwal, 2004). Whether diosgenin controls the expression of these gene products was also examined. Our results show that diosgenin abolished, in a time-dependent fashion, the TNF-induced expression of c-myc and cyclin D1 (Figure 5c).

Diosgenin potentiates the cytotoxic effects of TNF and chemotherapeutic drugs

The activation of NF-κB has been shown to inhibit TNF-induced apoptosis (Van Antwerp *et al*., 1996; Wang *et al*., 1996). Our results have suggested the potential of diosgenin to enhance the apoptosis induced by TNF and other cytotoxic agents through the suppression of NF-κB-regulated antiapoptotic gene products. We investigated whether suppression of NF-κB by diosgenin affects TNF and chemotherapeutic agent-induced apoptosis by MTT assay. Diosgenin enhanced the cytotoxic effects of TNF, paclitaxel, and doxorubicin (Figure 6).

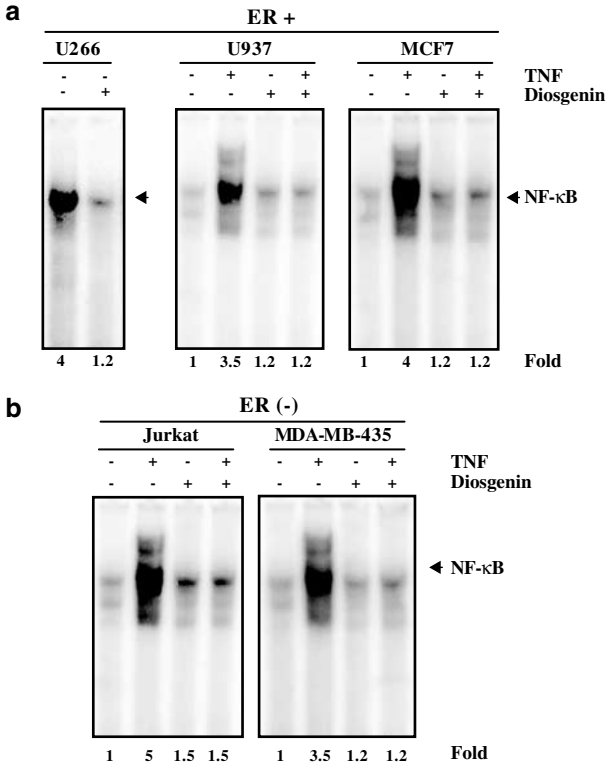

Figure 7 (**a**) Diosgenin inhibits constitutive and TNF-dependent NF-κB activation in estrogen receptor positive cells. Multiple myeloma (U266), leukemia (U937), and breast cancer (MCF-7) cells (2×10^6/ml) were preincubated with diosgenin as indicated for 24 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation, as described in Materials and methods. (**b**) Diosgenin inhibits TNF-dependent NF-κB activation in estrogen receptor-negative cells. Leukemia (Jurkat) and breast cancer (MDA-MB-435) cells (2×10^6/ml) were preincubated with diosgenin as indicated for 24 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF-κB activation, as described in Materials and methods.

Diosgenin suppresses the activation of NF-κB in both estrogen receptor (ER)-positive and ER-negative cells

Diosgenin is an estrogen-like compound and thus could mediate its effects through ER-dependent pathway (Scott *et al*., 2001). Whether suppression of TNF-induced NF-κB activation by diosgenin was mediated through ER, was investigated. To determine this, we examined the effect of diosgenin on both ER-positive (U266, U937, and MCF-7), and ER-negative (MDA-MB-435 and Jurkat) cell lines. We found that diosgenin pretreatment suppressed NF-κB activation in both ER-positive and ER-negative cell lines (Figure 7a and b). These results suggest that the NF-κB inhibitory activity of diosgenin is not mediated through the ER pathway.

Discussion

Fenugreek and roots of wild yam has been used in alternative medicine (Raju *et al*., 2004) for the treatment of diabetes (Madar *et al*., 1988; Sharma *et al*., 1990; Gupta *et al*., 2001), high cholesterol (Valette *et al*., 1984; Sauvaire *et al*., 1991), wounds, inflammation, and gastrointestinal ailments (Pandian *et al*., 2002; Raju *et al*., 2004). However, the mechanism of their action is poorly understood. In this study, we investigated the mechanism of action of diosgenin, a steroid sapogenin derived from fenugreek. We found that diosgenin inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis. Diosgenin also inhibited the proliferation of tumor cells and potentiated the apoptosis induced by TNF and chemotherapeutic agents. We further found that diosgenin downregulated the expression of antiapoptotic, proliferative, and angiogenic gene products. Diosgenin suppressed the activation of NF-κB induced by TNF through the inhibition of IKK activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, NF-κB reporter gene expression and suppression of Akt.

We demonstrate for the first time that diosgenin inhibited RANKL-induced osteoclastogenesis by blocking the differentiation of monocytes into osteoclasts. This result may explain the antiosteoporotic effects of diosgenin reported previously (Higdon *et al*., 2001; Scott *et al*., 2001; Yin *et al*., 2004). We also show that diosgenin suppressed TNF-induced invasion by tumor cells, and this inhibition correlated with the downregulation of MMP-9 and COX-2. The role of MMP-9 and COX-2 in invasion has been demonstrated earlier (Yamamoto *et al*., 1995; Attiga *et al*., 2000; Esteve *et al*., 2002).

Diosgenin inhibited the proliferation of tumor cells by stopping the cells from progressing to G1 phase. Our results are in agreement with earlier reports that have shown that diosgenin induced cell cycle arrest in tumor cells (Moalic *et al*., 2001; Liu *et al*., 2005). The cell cycle arrest correlated with a downregulation of TNF-induced cyclin D1 in diosgenin-treated cells. Cyclin D is required for the progression of cells from the G phase to the S phase (Matsushime *et al*., 1991).

As NF-κB has been implicated in osteoclastogenesis, tumor invasion, and proliferation and survival of tumor cells, we investigated whether diosgenin inhibited the activation of NF-κB. Our results show that diosgenin inhibited TNF-induced NF-κB activation. Our results are in complete disagreement with an earlier report (Moalic *et al*., 2001) where diosgenin was shown to activate NF-κB in osteosarcoma cells. This difference could be due to the nature of the cell line or the dose of diosgenin used in the earlier study. In that report, the mechanism of NF-κB activation was not examined, and the supershift assays were inconclusive. No kinetic study was performed. In our study, diosgenin blocked TNF-induced activation of IKK, which led to suppression of IκBα phosphorylation and subsequent degradation. The phosphorylation and nuclear translocation of p65 were also inhibited by diosgenin. Since Akt has been shown to activate IKK (Ozes *et al*., 1999), we examined whether diosgenin blocked Akt activity. We found that diosgenin suppressed TNF-induced activation of Akt. Our results indicate that diosgenin-induced inhibition of IKK activation may be mediated through the suppression of Akt activation.

This is also the first report to show that diosgenin inhibited NF-κB-regulated gene products involved in cell proliferation, antiapoptosis, invasion, and metastasis. The antiproliferative and apoptotic effects of diosgenin as reported earlier may also be mediated through the suppression of NF-κB. Our results also indicate that diosgenin can potentiate the apoptotic effects of TNF and chemotherapeutic agents. Cytokines and chemotherapeutic agents have been implicated in NF-κB activation (Arlt *et al*., 2001; Bottero *et al*., 2001; Tergaonkar *et al*., 2002) and upregulation of genes involved in cell survival (Shishodia and Aggarwal, 2004a). We found that most of the gene products involved in cell survival (e.g; bcl-2, Bcl-x_L, and IAP) were downregulated by diosgenin. Thus, diosgenin potentiated the apoptotic effects of TNF and chemotherapeutic agents, most likely through the suppression of NF-κB and NF-κB-regulated genes involved in cell survival. However, mechanisms other than NF-κB suppression cannot be ruled out. Our data indicates that diosgenin may mediate its anti-inflammatory, anticancer, and proapoptotic effects possibly through interruption of NF-κB pathway. Our study provides a rationale for combining diosgenin with cytokines and chemotherapeutic agents for the treatment of cancer.

Materials and methods

Materials

Diosgenin of 99% purity was obtained from Sigma (St Louis, MO, USA), dissolved in ethanol as a 10 mM stock solution, and stored at −20°C. Bacteria-derived recombinant human TNF purified to homogeneity with a specific activity of 5×10^7 U/μg was kindly provided by Genentech (South San Francisco, CA, USA). Penicillin, streptomycin, RPMI 1640, Iscove's modified DMEM medium (IMDM), fetal bovine serum (FBS), and the calcium phosphate transfection kit were obtained from Invitrogen (Grand Island, NY, USA). The following polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA): anti-p65, against the epitope corresponding to amino acids mapping within the amino terminal domain of human NF-κB p65; anti-p50, against a peptide 15 amino acids long mapping at the nuclear localization sequence region of NF-κB p50; anti-IκBα, against amino acids 297–317 mapping at the carboxy terminus of IκBα/MAD-3; anti-c-Rel, anti-cyclin D1 against amino acids 1–295, which represents full-length cyclin D1 of human origin; anti-MMP-9; anti-IAP1; anti-IAP2; anti-Bcl-2; and anti-Bfl-1/A1. Phosphospecific anti-IκBα (serine 32) and phosphospecific anti-p65 (serine 529) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences (San Diego, CA, USA). Anti-IKK-α, anti-IKK-β, and anti-FLIP antibodies were kindly provided by Imgenex (San Diego, CA, USA).

Cell lines

Human myeloid KBM-5 cells, mouse macrophage Raw 264.7 cells, and human embryonic kidney A293 cells were obtained from American Type Culture Collection (Manassas, VA, USA). KBM-5 cells were cultured in IMDM supplemented with 15% FBS. Raw 264.7 cells were cultured in DMEM/F-12 medium, and A293 cells were cultured in DMEM supplemented with 10% FBS. Human multiple myeloma (U266), leukemia (U937, Jurkat) and breast cancer (MCF-7) were cultured in RPMI supplemented with 10% FBS. Breast cancer (MDA-MB-435) cells were cultured in MEM with supplements. All media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Osteoclast differentiation assay

To determine whether diosgenin suppresses RANKL-induced osteoclastgenesis, we cultured RAW 264.7 cells, which can differentiate into osteoclasts after RANKL induction *in vitro* (Bharti *et al*., 2004). RAW 264.7 cells were cultured in 24-well dishes at a density of 1×10^4 cells per well and allowed to adhere overnight. The medium was then replaced, and the cells were pretreated with 10 μM diosgenin for 12 h and then treated with 5 nM RANKL. At days 4 and 5, the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression, a surrogate for RANKL expression, using an acid phosphatase kit (Sigma-Aldrich). The TRAP-positive multinucleated osteoclasts (>3 nuclei) in each well were counted.

Invasion assay

We assessed cell invasion because invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat Tumor Invasion system is a chamber that has a light-tight polyethelyene terephthalate membrane with 8-μm diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of 2.5×10^4 H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 10 μM diosgenin for 12 h and then stimulated with 1 nM TNF for a further 24 h in the presence of 1% FBS and diosgenin. The cells that invaded through the Matrigel (i.e., those that migrated to the lower chamber during incubation) were stained with 4 μg/ml Calcein AM (Molecular Probes) in PBS for 30 min at 37°C and scanned for fluorescence with a Victor 3 multiplate reader (PerkinElmer Life and Analytical Sciences, Boston, MA, USA); fluorescent cells were counted.

Flow cytometric analysis

To determine the effect of diosgenin on the cell cycle, KBM-5 cells were treated for different times, washed, and fixed with 70% ethanol. After incubation overnight at −20°C, cells were washed with PBS and then suspended in staining buffer (propidium iodide, 10 μg/ml; Tween-20, 0.5%; RNase, 0.1% in PBS). The cells were analysed using a FACS Vantage flow cytometer that uses CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA, USA). Gating was set to exclude cell debris, cell doublets, and cell clumps.

Cytotoxicity assay

The cytotoxic effects of diosgenin were determined by the MTT uptake method (Shishodia and Aggarwal, 2004a). Briefly, 5000 cells were incubated with diosgenin in triplicate in 96-well plates at 37°C. MTT solution was then added to each well. After a 2-h incubation at 37°C, extraction buffer (20% sodium dodecyl sulfate (SDS), 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and the OD was then measured at 570 nm using a 96-well multiscanner (Dynex Technologies, MRX Revelation, Chantilly, VA, USA).

Thymidine incorporation assay

The antiproliferative effects of diosgenin were also monitored by the thymidine incorporation method (Bharti *et al*., 2003). For this, 5000 cells in 100 μl of medium were cultured in triplicate in 96-well plates in the presence or absence of

diosgenin for 24 h. At 6 h before the completion of the experiment, cells were pulsed with 0.5 μCi (0.0185 MBq) [^{3}H]thymidine, and the uptake of [^{3}H]thymidine was monitored by means of a Matrix-9600 β-counter (Packard Instruments, Downers Grove, IL, USA).

Electrophoretic mobility shift assay (EMSA)
To assess NF-κB activation, we performed EMSA as described previously (Shishodia and Aggarwal, 2004a), with the following exceptions. Briefly, nuclear extracts prepared from treated cells (1×10^6/ml) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites), for 30 min at 37°C, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA **CTCACTTTC** CGCTG **CTCACTTTC** CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 min at 37°C before the complex was analysed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantitated using Imagequant software (Amersham, Piscataway, NJ, USA).

Western blot analysis
To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (Shishodia and Aggarwal, 2004a) and fractionated them by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by ECL reagent (Amersham). The bands obtained were quantitated using NIH Image (NIH, Bethesda, MD, USA).

IKK assay
To determine the effect of diosgenin on TNF-induced IKK activation, IKK assay was performed by a method we described previously (Shishodia and Aggarwal, 2004a), with the following exceptions. Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKKα and IKKβ and then treated with protein A/G-Sepharose beads (Pierce, Rockford, IL, USA). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate GST-IκBα (aa 1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS–PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKKα and IKKβ in each sample, 50 μg of whole-cell proteins was resolved on 7.5% SDS–PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKKα or anti-IKKβ antibody.

NF-κB-dependent reporter gene expression assay
The effect of diosgenin on NF-κB-dependent reporter gene transcription induced by TNF was analysed by SEAP assay as previously described (Shishodia and Aggarwal, 2004a), with the following exceptions. Briefly, A293 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMVFLAG1 DNA for 24 h. We then treated the cells for 12 h with diosgenin and then stimulated them with 1 nM TNF. The cell culture medium was harvested after 24 h of TNF treatment. Culture medium was analysed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA, USA) using a Victor 3 microplate reader (PerkinElmer).

Abbreviations

TNF, tumor necrosis factor; RANKL, receptor-activated nuclear factor-kappaB ligand; NF-κB, nuclear factor-kappaB; IκB, inhibitory subunit of NF-κB; SEAP, secretory alkaline phosphatase; IKK, IκBα kinase; COX-2, cyclooxygenase-2; LOX, lipoxygenase; MMP-9, matrix metalloproteinase-9; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate.

Acknowledgements

We thank Walter Pagel for a careful review of the manuscript. Dr Aggarwal is a Ransom Horne Jr Professor of Cancer Research. This work was supported in part by the Odyssey Program and the Theodore N Law Award for Scientific Achievement at The University of Texas MD Anderson Cancer Center (to SS). This work was also supported by the Clayton Foundation for Research (to BBA), a Department of Defense US Army Breast Cancer Research Program Grant (BC010610, to BBA), a PO1 Grant (CA91844) from the National Institutes of Health on lung chemoprevention (to BBA), and a P50 Head and Neck SPORE grant from the National Institutes of Health (to BBA).

References

Abu-Amer Y, Tondravi MM. (1997). *Nat Med* **3**: 1189–1190.
Aggarwal BB. (2003). *Nat Rev Immunol* **3**: 745–756.
Aggarwal BB. (2004). *Cancer Cell* **6**: 203–208.
Arlt A, Vorndamm J, Breitenbroich M, Folsch UR, Kalthoff H, Schmidt WE *et al*. (2001). *Oncogene* **20**: 859–868.
Attiga FA, Fernandez PM, Weeraratna AT, Manyak MJ, Patierno SR. (2000). *Cancer Res* **60**: 4629–4637.
Bharti AC, Donato N, Singh S, Aggarwal BB. (2003). *Blood* **101**: 1053–1062.
Bharti AC, Takada Y, Shishodia S, Aggarwal BB. (2004). *J Biol Chem* **279**: 6065–6076.
Bottero V, Busuttil V, Loubat A, Magne N, Fischel JL, Milano G *et al*. (2001). *Cancer Res* **61**: 7785–7791.
Corbiere C, Liagre B, Bianchi A, Bordji K, Dauca M, Netter P *et al*. (2003). *Int J Oncol* **22**: 899–905.
Corbiere C, Liagre B, Terro F, Beneytout JL. (2004). *Cell Res* **14**: 188–196.
Dasgupta B, Pandey VB. (1970). *Experientia* **26**: 475–476.

Djerassi C. (1992). *Science* **258**: 203–204.
Esteve PO, Chicoine E, Robledo O, Aoudjit F, Descoteaux A, Potworowski EF *et al*. (2002). *J Biol Chem* **277**: 35150–35155.
Ghosh S, May MJ, Kopp EB. (1998). *Annu Rev Immunol* **16**: 225–260.
Gupta A, Gupta R, Lal B. (2001). *J Assoc Physicians India* **49**: 1057–1061.
Heble MR, Narayanaswami S, Chadha MS. (1967). *Science* **161**: 1145.
Hibasami H, Moteki H, Ishikawa K, Katsuzaki H, Imai K, Yoshioka K *et al*. (2003). *Int J Mol Med* **11**: 23–26.
Higdon K, Scott A, Tucci M, Benghuzzi H, Tsao A, Puckett A *et al*. (2001). *Biomed Sci Instrum* **37**: 281–286.
Leger DY, Liagre B, Corbiere C, Cook-Moreau J, Beneytout JL. (2004). *Int J Oncol* **25**: 555–562.
Liagre B, Vergne-Salle P, Corbiere C, Charissoux JL, Beneytout JL. (2004). *Arthritis Res Ther* **6**: R373–83.
Liotta LA, Thorgeirsson UP, Garbisa S. (1982). *Cancer Metastasis Rev* **1**: 277–288.
Liu MJ, Wang Z, Ju Y, Wong RN, Wu QY. (2005). *Cancer Chemother Pharmacol* **55**: 79–90.
Madar Z, Abel R, Samish S, Arad J. (1988). *Eur J Clin Nutr* **42**: 51–54.
Matsushime H, Roussel MF, Ashmun RA, Sherr CJ. (1991). *Cell* **65**: 701–713.
Miyamoto S, Maki M, Schmitt MJ, Hatanaka M, Verma IM. (1994). *Proc Natl Acad Sci USA* **91**: 12740–12744.
Moalic S, Liagre B, Corbiere C, Bianchi A, Dauca M, Bordji K *et al*. (2001). *FEBS Lett* **506**: 225–230.
Nappez C, Liagre B, Beneytout JL. (1995). *Cancer Lett* **96**: 133–140.
Nasuhara Y, Adcock IM, Catley M, Barnes PJ, Newton R. (1999). *J Biol Chem* **274**: 19965–19972.
Ondeykal JG, Herath KB, Jayasuriya H, Polishook JD, Bills GF, Dombrowski AW *et al*. (2005). *Mol Divers* **9**: 123–129.
Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. (1999). *Nature* **401**: 82–85.
Pandian RS, Anuradha CV, Viswanathan P. (2002). *J Ethnopharmacol* **81**: 393–397.
Puri HS, Jefferies TM, Hardman R. (1976). *Planta Med* **30**: 118–121.
Raju J, Patlolla JM, Swamy MV, Rao CV. (2004). *Cancer Epidemiol Biomarkers Prev* **13**: 1392–1398.
Sauvaire Y, Ribes G, Baccou JC, Loubatieeres-Mariani MM. (1991). *Lipids* **26**: 191–197.
Scott A, Higdon K, Tucci M, Benghuzzi H, Puckett A, Tsao A *et al*. (2001). *Biomed Sci Instrum* **37**: 13–18.
Segal R, Milo-Goldzweig I, Zaitschek DV. (1977). *Lloydia* **40**: 604.
Sharma RD, Raghuram TC, Rao NS. (1990). *Eur J Clin Nutr* **44**: 301–306.
Shishodia S, Aggarwal BB. (2004a). *J Biol Chem* **279**: 47148–47158.
Shishodia S, Aggarwal BB. (2004b). *Biochem Pharmacol* **68**: 1071–1080.
Tergaonkar V, Pando M, Vafa O, Wahl G, Verma I. (2002). *Cancer Cell* **1**: 493–503.
Valette G, Sauvaire Y, Baccou JC, Ribes G. (1984). *Atherosclerosis* **50**: 105–111.
Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. (1996). *Science* **274**: 787–789.
Wang CY, Mayo MW, Baldwin Jr AS. (1996). *Science* **274**: 784–787.
Wang SL, Cai B, Cui CB, Liu HW, Wu CF, Yao XS. (2004). *J Asian Nat Prod Res* **6**: 115–125.
Yamada T, Hoshino M, Hayakawa T, Ohhara H, Yamada H, Nakazawa T *et al*. (1997). *Am J Physiol* **273**: G355–64.
Yamamoto K, Arakawa T, Ueda N, Yamamoto S. (1995). *J Biol Chem* **270**: 31315–31320.
Yin J, Tezuka Y, Kouda K, Le Tran Q, Miyahara T, Chen Y *et al*. (2004). *Planta Med* **70**: 220–226.
Zhong H, Voll RE, Ghosh S. (1998). *Mol Cell* **1**: 661–671.