The Biphasic Role of NF-κB in Progression and Chemoresistance of Ovarian Cancer

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Abstract

\textbf{Purpose:} NF-κB is a transcription factor known to promote tumorigenesis. However, NF-κB is also known to be proapoptotic and may potentially function as a tumor suppressor, although such a functional role has not been extensively investigated in human cancer.

\textbf{Experimental Design:} A dominant-negative mutant of IκB\textsubscript{a} with mutations at S32A and S36A was used to inhibit the function of NF-κB in ovarian cancer cell lines. The transcription ability, tumorigenesis, apoptosis, and drug sensitivity were examined in derivative cell lines in comparison with parental cells. We also analyzed the association of nuclear expression of NF-κB p65 with patient survival in an ovarian cancer tissue array.

\textbf{Results:} We show that NF-κB functions as a tumor suppressor in four ovarian cancer cell lines, but it functions as an oncogene in their aggressive chemoresistant isogenic variants. NF-κB can exert its proapoptotic or antiapoptotic effect by activating or repressing mitogen-activated protein kinase (MAPK) phosphorylation in parental or aggressive chemoresistant variant cell lines. We also show that the nuclear accumulation of p65 in epithelial cancer tissue is associated with a good response to chemotherapy and can predict longer overall survival for patients with ovarian cancer.

\textbf{Conclusions:} Our data provide strong evidence that NF-κB can function as a biphasic regulator, either suppressing or enhancing ovarian cancer growth through the regulation of MAPK and cellular apoptosis.

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Introduction

NF-κB is a ubiquitous transcription factor that controls the expression of various genes associated with immune responses, the cell cycle, and apoptosis (1, 2). Dysregulation of NF-κB plays an important role in many disease processes, including inflammatory and autoimmune diseases, viral infection, and cancers (3). NF-κB is often found in its inhibited form bound to IκB in the cytoplasm and can be activated by upstream events. One such event is the binding of TNF-α to its receptor, which results in the phosphorylation of IκB by IκB kinase (IKK). IκB is then ubiquitinated and degraded by the 26S proteasome and NF-κB (p50, p52, and p65) is released. The free NF-κB subunits translocate to the nucleus and binds to specific promoters to activate the transcription of downstream target genes (4). However, a super repressor of IκB\textsubscript{a} with mutations at S32A and S36A (IκB\textsubscript{aM}) can competitively bind to the NF-κB subunits and block the phosphorylation of endogenous IκB\textsubscript{a} by IKK and the translocation of p65, which inhibits the normal activity of NF-κB (5–7). Therefore, IκB\textsubscript{aM} has been widely used to study the function of NF-κB in various cells and tissues (8–10).

The oncogenic function of NF-κB, which has been well documented in many cancers, is largely due to its effect on activating multiple downstream target genes involved in antiapoptosis, cell-cycle progression, and angiogenesis (11–13). However, other studies show that NF-κB can function as a tumor suppressor to induce apoptosis (14, 15). For example, activation of NF-κB promotes p53-mediated apoptosis (16) and the activated NF-κB is a predictor of better prognosis in gastric cancer patients (17). Inhibition of NF-κB results in spontaneous squamous cell carcinomas (18) and skin cancer (19). Blocking the activation of NF-κB by IκB\textsubscript{aM} can synergize with RAS to induce human keratinocyte malignancy through increased CDK4 expression (20). In addition, NF-κB p65 suppresses the transcription of antiapoptotic genes in ultraviolet C (21) or daunorubicin/doxorubicin-induced apoptosis (22).
Ovarian cancer remains the most common cause of death from gynecologic malignancies. Although 80% of patients respond to initial treatment with platinum-based chemotherapy, about 70% of these patients have disease recurrence and die from their disease. Because primary ovarian cancer is removed by surgery, recurrent ovarian cancer, particularly, cisplatin-resistant ovarian cancer, is a major cause of death in patients (23, 24). In ovarian cancer, the increased activity of NF-κB has been reported to be a predictor of poor disease progression (25, 26) and to confer resistance to cisplatin-induced apoptosis (27). Activation of NF-κB signaling by inhibitor of NF-κB kinase (IKK) increases aggressiveness of ovarian cancer (28). More recently, proteomic analysis identified RelA and STAT5 as 2 major proteins associated with resistance to chemotherapeutic agents in ovarian high-grade serous carcinomas (29). Blocking p65 NF-κB activity with IkBαM in SKOV3.ip1 and HEYA8 ovarian cancer cells can reduce angiogenesis and tumor growth in xenograft mice (30). One study found that activation of the NF-κB signal pathway reduces paclitaxel-induced apoptosis in ovarian cancer cells (31), whereas other studies have shown that NF-κB mediates paclitaxel-induced apoptosis in ovarian cancer cells (32). Furthermore, treatment of epithelial ovarian cancer cells with carboplatin upregulates expression of level of NF-κB, which may be associated with proapoptosis (33, 34). These data indicate that the role of NF-κB in ovarian cancer varies depending on the experimental system and tumor models.

In this study, we investigated the role of NF-κB in ovarian cancer cell lines and their isogenic aggressive and chemoresistant variants and ovarian cancer from patients. Our results show that NF-κB functions as a tumor suppressor in ovarian cancer but can be reprogrammed to become an oncogene in aggressive and chemoresistant ovarian cancer cells.

Materials and Methods

Cell lines, media, and plasmids
The high-grade serous ovarian cancer cell lines SKOV3, HEY, SKOV3.ip1, HEYA8, OVCA3, and OVCA433 were maintained in Eagle’s minimum essential medium (Lonza Walkersville, Inc.). SKOV3.ip1 and HEYA8, the isogenic cell lines of SKOV3 and HEY, are resistant to carboplatin or paclitaxel. The dominant-negative mutant of IkBα (protein phosphatase 1α) (protein phosphatase 1α) and control siRNAs were purchased from Santa Cruz Biotechnology.

Retroviral production and infection
The protocol for retroviral production and infection to establish the following virus-containing cell lines was carried out as described previously (36): SKOV3/vector, HEY/vector, SKOV3/IkBαM, HEY/IkBαM; SKOV3/ip1/vector, HEYA8/vector, and SKOV3/ip1/IkBαM, HEYA8/IkBαM; OVCA3/vector, OVCA433/vector, and OVCA3/IkBαM, OVCA433/IkBαM.

Electrophoretic mobility shift assay and luciferase reporter assay
Electrophoretic mobility shift assay (EMSA) was carried out using nuclear extracts prepared as described previously (37). NF-κB DNA consensus sequences (sc-2505) and mutant sequences (sc-2511) were obtained from Santa Cruz Biotechnology. DNA oligos labeled with 32P-dATP by T4 polynucleotide kinase were used as the hot probes, and anti-p65 antibody (BD Pharmingen) was used to detect the p65-DNA complex. The assay was carried out according to a previously published method (37).

For the NF-κB transcription assay, cells were seeded into 24-well plates, precultured to 75% confluence, and then transfected with an HIV promoter–driven luciferase cDNA plasmid as described previously (37). Luciferase activity was tested using a kit from Promega. The firefly luciferase activity was normalized to Renilla luciferase activity. The assay was repeated 3 times in duplicate.

Tumor formation in nude mice
Animal assays were done following the protocol approved from institutional committee for animal experiments. Equal numbers of cells (5 × 10⁶ cells/injection for OVCA3, OVCA433 cells; 1 × 10⁶/injection for SKOV3, HEY, SKOV3.ip1, HEYA8 cells transfected with IkBαM or vector) were harvested by trypsinization, washed twice with PBS, resuspended in 0.15 mL of PBS, and injected either
I and repeated 3 times. Representing an early apoptotic population (Annexin V was calculated from the peaks (M2) in the histogram were analyzed with a FACStation flow cytometer equipped with standard methods for Western blotting (37). Equal amounts of protein (30 μg) were analyzed using standard methods for Western blotting (37). Antibodies to IkBα, pIkBα (Ser32/36), p65, PP1α, protein phosphatase 2A (PP2A), MEK1/2, ERK1/2, pMEK1/2 (Ser217/221), pERK1/2 (Thr202/Tyr204), BAK, Bid, Bcl-2, Bcl-xL, JNK1/2, and pJNK1/2 (Thr183/Tyr185) were purchased from Cell Signaling Inc. β-Actin (Sigma-Aldrich) was used as a loading control.

Apoptosis detection

Cells were freshly harvested by trypsinization after washing 3 times with PBS. The cells were then fixed with 75% alcohol at −20°C for 3 days and stained with Annexin V and propidium iodide (PI) according to the manufacturer’s instructions in the Annexin V-fluorescence apoptosis detection kit I (BD Biosciences Pharmingen). The stained cells were analyzed with a FACStation flow cytometer equipped with CellQuest software. The percentage of apoptotic cells was calculated from the peaks (M2) in the histogram representing an early apoptotic population (Annexin V+/PI−; ref. 37). The experiment was carried out in duplicate and repeated 3 times.

For the TUNEL (terminal deoxynucleotide transferase dUTP nick-end labeling) assay, xenograft tumor tissues from nude mice inoculated with SKOV3/IkBαM, HEY/IkBαM, SKOV3.ip1/IkBαM, HEYA8/IkBαM, OVCAR3/IkBαM, or OVC4433/IkBαM cells or their vector-transfected controls were formalin-fixed and paraffin-embedded. The slides containing the tissue sections were stained with an Apo-BrdU in situ DNA fragmentation assay kit (Medical & Biological Laboratories) according to the manufacturer’s instructions. The total number of fluorescent cells, as determined by fluorescent microscopy, was used as an indication of apoptosis. For quantification, the total number of cells and the number of apoptotic cells in each field were counted and the percentage of apoptotic cells was calculated. A minimum of 1,000 cells per slide were counted in 8 to 12 randomly selected microscopic views. Assays were repeated 3 times.

Treatment of cells with chemicals

Carboplatin (500–0.5 μmol/L dilution) and paclitaxel (50–0.5 nM/L dilution) were used to determine the half-maximal inhibitory concentration (IC50) values in SKOV3, HEY, SKOV3.ip1, HEYA8, OVCAR3, and OVCA433 cells in the presence or absence of IkBαM. The MTT (American Type Culture Collection) assay was used to measure cell viability at 570 nm after treatment with these drugs and a recovery of 24 hours. OVCAR3 and OVCA433 cells were treated with PS1145 (20 μmol/L) to observe the alterations in the levels of signal molecules associated with NF-κB activation. Dimethyl sulfoxide (DMSO) was used as a control. Cells were harvested after 4 and 8 hours of treatment. To inhibit the activity of PP1α, okadaic acid (20 nmol/L) was used to treat IkBαM-transfected cells for 12 and 24 hours. DMSO treatment was used as a control. Assays were repeated 3 times.

Patient tissue specimens

The use of tissue blocks and medical record reviews were approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. Patients had been treated with either cisplatin or carboplatin by the treating physicians, and the selection of patient tissues was not based on the treatments. Follow-up information was updated through March 2010 by reviewing medical records and the U.S. Social Security Index.

The randomly selected formalin-fixed, paraffin-embedded tissues included normal ovarian tissues (n = 7), normal fallopian tube (n = 5), and high-grade serous ovarian carcinomas (n = 324) and were not matched. Tumor sample collection and tissue microarray construction have been described elsewhere (37). Briefly, ovarian tissue microarray blocks were selected by 2 gynecologic pathologists (I.L. and D.G.R.), who reviewed hematoxylin and eosin-stained sections and constructed by taking core samples from morphologically representative areas of paraffin-embedded tumor tissues and assembling them on a recipient paraffin block.

Immunohistochemical staining and analysis

Immunohistochemical staining for NF-κB p65 (Abcam Inc.) was carried out using avidin–biotin peroxidase methods, as described elsewhere (37). NF-κB p65 intensity was scored by scanning tissue microarray slides with a computerized imaging system (Ariol SL-50; Applied Imaging), and the data were reported automatically to a linked clinical database. The cutoff point for the nuclear NF-κB p65 score to reach statistical significance in terms of overall and disease-free survival was analyzed using X-tile software (The Rimm Lab at Yale University; http://www.tissuearray.org/rimmlab), as described elsewhere (38).

Disease-specific survival rates were calculated as the percentage of the subjects who survived with disease for a defined period, reported as time since diagnosis or treatment, and only deaths from the disease were counted. Patients were divided into chemosensitive and chemoresistant groups according to their response to cisplatin-based therapy, which was documented on the patients’ charts. The platinum chemosensitive group included patients who did not show relapse or progression for more than 6 months with complete or partial response. The other who underwent relapse or progression before 6 months with complete or partial clinical response were included in the platinum-resistant group.
Statistical analysis

The number of mice (sample size) required to reach statistical significance was determined in preliminary pilot studies that used the following formula (39): n = 16 × (SD/difference in mean tumor volume)² + 1. Results of that pilot study indicated that 6 mice would be required to detect differences in tumor size, with 80% power at a P value of less than 0.05. Statistical analysis was carried out using Fisher’s exact test at different time points for the mean tumor sizes of each group.

X-tile software was used to find the optimal cutoff point. The prognostic significance was determined using Monte Carlo simulations, a robust statistical analysis that is appropriate for generation of optimal cutoff points for continuous data (38). Differences in proportion of p65 nuclear expression in tumor tissues were evaluated by the m² or Fisher’s exact test as appropriate. The correlation between p65 expression in tissue arrays (based on the p65 immunostaining intensity scores) and patient survival was analyzed by the Kaplan–Meier method by using SPSS 17.0 software (SPSS Inc.). Disease-specific survival rates were calculated using the Kaplan–Meier method and compared by the log-rank test. Clinical correlation in terms of p65 expression and patient survival was carried out by excluding missing data. Cox proportional hazards regression models in STATISTICA software (SAS Language Reference, version 8; SAS Institute, Inc.) were used for univariate and multivariate analyses of survival. Results were considered statistically significant if the P value was less than 0.05. All statistical tests were 2-sided.

Results

Constitutive activation of NF-κB inhibits tumor growth in ovarian cancer cells but has an opposite effect in isogenic aggressive and chemoresistant ovarian cancer cells

To characterize the function of NF-κB in ovarian cancer cells, we first tested its effect on tumor growth in 2 ovarian cancer cell lines, SKOV3 and HEY, and 2 isogenic aggressive and chemoresistant ovarian cancer cell lines derived from SKOV3 and HEY, named SKOV3.ip1 and HEYA8, respectively. These 2 cell lines were derived from ascites from xenograft mice that had been injected with SKOV3 or HEY cells and displayed more aggressive growth or metastatic ability than the parental cell lines (40–42). Transfection of these cell lines with IκBαM reduced the DNA binding and gene transcription activity of NF-κB, as indicated by EMSA (Fig. 1A) and a luciferase reporter assay (Fig. 1B), respectively. We found that nude mice injected with IκBαM-transfected SKOV3 and HEY cells had more subcutaneous tumor growth than those injected with control cells (Fig. 1C). IκBαM-mediated inhibition of NF-κB reduced the levels of cellular apoptosis in SKOV3 and HEY cells, as detected by Annexin V staining, and in xenograft mouse tissues derived from SKOV3 and HEY cells, as measured by an Apo-BrdU-labeled in situ DNA fragmentation TUNEL assay (Fig. 1D and E). However, the introduction of IκBαM suppressed tumor growth in SKOV3.ip1- and HEYA8-injected mice (Fig. 1C) and enhanced apoptosis in SKOV3.ip1 and HEYA8 cells and xenograft tissues derived from these cells (Fig. 1D and E), which is consistent with the data from previous report (30). In addition, peritoneal injection of mice with SKOV3/IκBαM and HEY/IκBαM cells resulted in tumor nodules that were bigger than those in mice injected with SKOV3/vector and HEY/vector cells, but the mice injected with SKOV3.ip1/IκBαM and HEYA8/IκBαM cells had smaller tumor nodules than control mice injected with SKOV3.ip1/vector and HEYA8/vector cells (Supplementary Fig. S1). These results suggest that in SKOV3.ip1 and HEYA8 cells NF-κB is oncogenic and suppresses apoptosis, in contrast to its function as a tumor suppressor in SKOV3 and HEY cells. Our results confirmed the previously reported function of NF-κB as an oncogene in SKOV3.ip1 and HEYA8 cells; however, the results that NF-κB functions as a tumor suppressor in the isogenic parental cell lines SKOV3 and HEY (less aggressive variants) were unexpected and have not been reported.

To validate this conclusion, we further investigated the function of NF-κB in 2 additional ovarian cancer cell lines, OVCAR3 and OVCA433. EMSA analysis indicated that the DNA-binding activity of p65 NF-κB was markedly reduced in IκBαM-transfected cells compared with control cells (Fig. 2A). In addition, a luciferase reporter assay showed the markedly reduced transcriptional activity of NF-κB in wild-type promoter-transfected cells expressing IκBαM (Fig. 2B). We also analyzed in vivo tumor growth by injecting cells into nude mice and found that both OVCAR3 and OVCA433 cells expressing IκBαM resulted in larger subcutaneous tumors (Fig. 2C and D) and more peritoneal nodules (Fig. 2D) than did control cells. Annexin V staining of cultured OVCAR3/IκBαM and OVCA433/IκBαM cells and Apo-BrdU-labeled in situ DNA fragmentation TUNEL assays with xenograft mouse tissues derived from these cells revealed that the level of apoptosis was reduced after knockdown of NF-κB with IκBαM (Fig. 2E–H). These data strongly suggest that NF-κB functions as a tumor suppressor in these 2 ovarian cancer cell lines, similar to the results observed in SKOV3 and HEY cells. Thus, we have shown in 4 independent ovarian cancer cell lines that NF-κB has a tumor suppressive role, suggesting that NF-κB is a biphasic regulator that can act as either a tumor suppressor or an oncogene in ovarian cancer, possibly through regulation of apoptosis signal pathway.

NF-κB–mediated chemosensitivity in ovarian cancer cells can be reversed by ectopic expression of IκBαM

NF-κB is known to be involved in chemoresistance, primarily through its effect on antiapoptosis (43). However, activation of NF-κB also contributes to chemosensitivity (44). Because our data showed that NF-κB can function as a tumor suppressor, we examined whether the NF-κB can mediate the chemosensitivity in these ovarian cancer cell lines. We first compared the chemosensitivity of parental ovarian cancer cells (SKOV3 and HEY) with that of recurrent ovarian cancer cells (SKOV3.ip1 and HEYA8),
Figure 1. Tumor growth and apoptosis following introduction of IκBαM in ovarian cancer cell lines. A, EMSA showing reduced DNA-binding activity of NF-κB in the ovarian cancer cell lines SKOV3, HEY, SKOV3.ip1, and HEYA8 expressing IκBαM. B, luciferase reporter assays showing the reduced transcriptional activity of NF-κBαM-transfected cells. Mt, mutant; Wt, wild type. Error bars, 95% CIs. C, xenograft tumor growth in nude mice injected with ovarian cancer cells with or without IκBαM expression. (P < 0.05). D, percentage of apoptotic cells measured by Annexin V staining of cells with or without IκBαM expression (left) and measured by TUNEL assays in xenograft tumor tissues (middle and right). Error bars, 95% CIs. E, representative images of xenograft tumor tissue showing apoptosis, which is indicated by DNA breaks (green) detected by an Apo-BrdU (green) in situ DNA fragmentation assay (×400, TUNEL). PI, red.
Figure 2. Tumor growth and apoptosis following ectopic expression of IκBαM in the ovarian cancer cell lines OVCAR3 and OVCA433. A, EMSA showing that the binding of NF-κB to its promoter DNA consensus sequence was reduced in IκBαM-transfected cells. B, luciferase reporter assays showing the reduced transcriptional activity of NF-κB. Error bars, 95% CIs. Mutant; WT, wild type. C, tumor growth in nude mice injected subcutaneously with OVCAR3 and OVCA433 cells. Mice injected with IκBαM-transfected cells displayed enhanced tumor burden. Error bars, 95% CIs. D, average number of tumor nodules in mice injected with ovarian cancer cells in the presence and absence of IκBαM. The number of nodules was higher in mice injected with IκBαM-treated cells than in those injected with control cells. Error bars, 95% CIs. E and F, Annexin V staining showing a reduction in the percentage of apoptotic cells after knockdown of NF-κB. Error bars, 95% CIs. G and H, analysis of apoptosis in xenograft mouse tumor tissues using a TUNEL assay with Apo-BrdU (green) in situ fragmentation (×400). Error bars, 95% CIs. PI, red.

using carboplatin and paclitaxel, 2 drugs commonly used for ovarian cancer treatment, in the presence or absence of IκBαM. By determining the IC_{50} of carboplatin and paclitaxel in these cells, we found that SKOV3.ip1 and HEYA8 cells have much higher IC_{50} values than primary SKOV3 and HEY cells (Fig. 3A). The IC_{50} values for carboplatin in
Figure 3. Chemosensitivity in ovarian cancer cells with or without IκBαM expression. A, IC50 values for carboplatin or paclitaxel in ovarian cancer cells (SKOV3 and HEY), and recurrent ovarian cancer cells (SKOV3.ip1 and HEY/A8). Error bars, 95% CIs. Cell viability was measured by an MTT assay after cells were treated with either (B) carboplatin or (C) paclitaxel at the concentrations indicated. OVCAR3/IκBαM and OVCA433/IκBαM cells were more viable than control cells. Error bars, 95% CIs. D, IC50 values for carboplatin and paclitaxel. The IC50 value was increased by up to 4 times after cells were transfected with IκBαM. Error bars, 95% CIs.
SKOV3.ip1 and HEYA8 cells were 180 and 232 μmol/L, respectively, nearly 4 times higher than those in vector-transfected SKOV3 (49 μmol/L) and HEY (60 μmol/L) cells. In addition, SKOV3/IκBαM and HEY/IκBαM cells had higher IC_{50} values than their vector controls, but SKOV3.ip1/IκBαM and HEYA8/IκBαM cells were more sensitive to the drugs than their control cells (Fig. 3A). These results show that NF-κB increases the sensitivity to carboplatin and paclitaxel in SKOV3 and HEY cells, whereas it increases the resistance of SKOV3.ip1 and HEYA8 cells to these agents.

We further confirmed the role of NF-κB as a sensor of apoptosis in OVCAR3 and OVCA433 cells. We observed that after treating cells with carboplatin and paclitaxel, the number of viable cells, as measured by an MTT assay, was greater in IκBαM-transfected cells than in control cells (Fig. 3B and C). The IC_{50} values were at least 4 times higher in IκBαM-transfected OVCAR3 and OVCA433 cells than in control cells (Fig. 3D). Taken together, these data show that NF-κB is required to sensitize ovarian cancer cells to chemotherapeutic agents but that in aggressive and recurrent ovarian cancer cells, it blocks apoptosis and increases drug resistance.

NF-κB–induced apoptosis is associated with phosphorylation of MAPKs

To explore the underlying mechanism by which NF-κB functions as a tumor suppressor, we examined the expression of signaling molecules involved in the MAPK and cellular apoptosis pathways. We found an increase in pMEK1/2 (Ser217/221) expression in SKOV3.ip1/IκBαM cells but not in SKOV3, HEY, and HEYA8 cells transfected with IκBαM (Fig. 4). Interestingly, the phosphorylation of ERK1/2 (Thr202/Tyr204) was reduced in SKOV3/IκBαM and HEY/IκBαM cells but was increased in SKOV3.ip1/IκBαM and HEYA8/IκBαM cells. The phosphorylation of JNK1/2 (Thr183/Tyr185) was increased in SKOV3.ip1/IκBαM and HEYA8/IκBαM cells but not in SKOV3/IκBαM and HEY/IκBαM cells (Fig. 4). In addition, the levels of the apoptotic proteins BAK and Bid were not changed in SKOV3/IκBαM and HEYA8/IκBαM cells compared with control cells. The level of the antiapoptotic protein Bcl-xL

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Figure 4. Altered MAPK signaling and apoptosis are associated with inhibition of NF-κB activity. Detection of MAPKs and apoptosis-associated proteins in ovarian cancer cells with or without IκBαM by Western blotting. β-Actin was used as a loading control.
was increased in SKOV3/IKBaM and HEY/IKBaM cells, whereas no changes in the level of Bcl-2 were visualized. The expression of Bid, but not BAK, was increased in both SKOV3.ip1/IKBaM and HEYA8/IKBaM cells, and the expression of Bcl-2 and Bcl-xL was reduced in SKOV3.ip1/IKBaM cells but not in HEYA8/IKBaM cells (Fig. 4). These results show that the phosphorylation of ERK1/2 (Thr202/Tyr204) may mediate the function of NF-kB in ovarian cancer cells. Blocking NF-kB function in ovarian cancer cells may promote the dephosphorylation of ERK1/2, which reduces the chemosensitivity of the cells by increasing the levels of cellular antiapoptosis proteins. However, inhibiting NF-kB with IxBaM in aggressive and chemo-resistant ovarian cancer cells seems to enhance the phosphorylation of ERK1/2, leading to increased chemosensitivity.

To further confirm the signaling pathways involved in NF-kB–mediated tumor suppression, we measured the expression of the signaling proteins described earlier in OVCAR3 and OVCA433 cells in the presence or absence of IxBaM (Fig. 4, right panel). We found that both pMEK1/2 and pERK1/2 levels were reduced after inhibiting NF-kB activity with IxBaM whereas the pIJK1/2 level was unchanged. In addition, the level of the antiapoptotic protein Bcl-2 was dramatically increased in both OVCAR3 and OVCA433 cells transfected with IxBaM and the antiapoptotic protein Bcl-xL was increased in OVCAR3 cells transfected with IxBaM (Fig. 4, right panel). No changes in the levels of the proapoptotic proteins BAK and Bid were observed in these cells. These results show that the NF-kB–mediated tumor suppression pathway may be regulated by the MAPK/ERK signaling pathway, and this tumor suppression pathway is conserved in the 4 independent ovarian cancer cell lines from different genetic backgrounds.

**PP1α regulates NF-kB–mediated apoptosis through dephosphorylation of MAPK in chemosensitive ovarian cancer cells**

Despite the fact that IxBaM has been commonly used to block NF-kB activation by different investigators, we wanted to verify whether the changes observed in apoptosis-related proteins and phosphorylation of MEK1/2 and ERK1/2 in the ovarian cancer cells transfected with IxBaM were caused by the specific inhibition of NF-kB. We first tested the inhibitory effect of NF-kB on nuclear translocation by IxBaM. The expression of NF-kB p65 was examined in cell fractionation (nuclear and cytosolic) after cells were treated with TNF-α. As shown in Supplementary Figure S2, nuclear p65 was increased in vector-transfected control cells after treatment of TNF-α but was undetectable in IxBaM-transfected cells before and after TNF-α treatment, showing that nuclear to cytoplasmic translocation is blocked in IxBaM-transfected cells. Thus, we measured the levels of these molecules in OVCAR3 and OVCA433 cells treated with either NF-kB p65 siRNA or the NF-kB signal–specific inhibitor PS1145. Knockdown of p65 with siRNA in OVCAR3 and OVCA433 cells reduced the levels of phosphorylated MEK1/2 and ERK1/2 and increased the expression of Bcl-2 compared with control siRNA–treated cells (Fig. 5A). Treating cells with PS1145 led to decreased phosphorylation of IxBa at serine 32/36, which increased the levels of Bcl-2 and reduced the levels of phosphorylated ERK1/2, but not MEK1/2, at 4 or 8 hours after treatment (Fig. 5B). These results show that inhibiting NF-kB by targeting p65 with siRNA or an NF-kB–specific inhibitor induces alterations in the apoptosis and MAPK pathways that are similar to those induced by IxBaM in ovarian cancer cells. In addition, we observed that the level of PP1α, but not PP2A, was increased in p65 siRNA- and PS1145-treated cells compared with control siRNA- and DMSO-treated cells (Fig. 5A and B). Because MAPK phosphorylation may be regulated by protein phosphatases (45), we also analyzed the expression of PP1α and PP2A in IxBaM-transfected cells, which revealed that the level of PP1α, but not PP2A, was increased as the expression of pMEK1/2 and pERK1/2 was decreased (Fig. 5C). These data indicate that PP1α may be involved in the regulation of MAPK phosphorylation in these ovarian cancer cell lines.

We next tested whether the phosphorylation of MEK1/2 and ERK1/2 was directly regulated by PP1α in our model ovarian cancer cells by using PP1α-specific siRNA or okadaic acid either to knockdown or to inhibit PP1α activity, respectively. Silencing PP1α expression with siRNA (Fig. 5D) or inhibiting PP1α activity with okadaic acid (Fig. 5E) dramatically increased the levels of the phosphorylated MEK1/2 and ERK1/2, leading to reduced Bcl-2 expression. These data indicate that NF-kB–mediated apoptosis in ovarian cancer is likely associated with the phosphorylation of MEK1/2 and ERK1/2, which is regulated by PP1α.

**Nuclear localization of NF-kB is associated with favorable prognoses for ovarian cancer patients**

Although it has been reported that NF-kB is a poor prognostic indicator in ovarian cancer (25, 26), our results in this study suggested that NF-kB may function as a tumor suppressor in ovarian cancer. Therefore, we further analyzed by immunostaining a tissue microarray consisting of 324 ovarian cancer cases. We found that the nuclear expression of p65 was low (≤12%, nuclear positive tissues) in 19.4% of cases (63/324) but high (≥12%, nuclear positive tissues) in 80.6% of cases (261/324), whereas the NF-kB p65 nuclear expression was largely absent in normal ovarian and fallopian tube epithelial cells. The correlation between clinicopathologic characteristics and NF-kB p65 nuclear expression is shown in Table 1. No correlation was found between p65 nuclear expression and International Federation of Gynecology and Obstetrics (FIGO) stage, family history, age at diagnosis, clinical response, or presence of ascites ($P > 0.05$). However, patients who had progressive disease (39.8%) tended to have lower expression of NF-kB than those who relapsed (43.8%: Table 1, footnote a, $P = 0.03$). We did not find a significant statistical correlation between patients with and without disease recurrence or between patients with progressive disease.
Figure 5. Detection of p65, pIkBα, pMEK1/2, pERK1/2, Bcl2, PP1α, and PP2A by Western blotting. A and B, OVCAR3 and OVCA433 cells were treated with either p65 siRNA (A) or PS1145 (B). C, levels of PP1α and PP2A in ovarian cancer cells with or without IkBαM. D and E, OVCAR3/IkBαM and OVCA433/IkBαM cells were treated with PP1α siRNA (D) or okadaic acid (E).
### Table 1. Association of NF-κB p65 expression with patient characteristics

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<td>137 (83)</td>
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<td>6 (85.7)</td>
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<td>261 (80.6)</td>
<td>324</td>
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<td><strong>Age at diagnosis, y</strong></td>
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<td>33 (18.8)</td>
<td>143 (81.2)</td>
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<td><strong>Level of debulking surgery</strong></td>
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<td>142 (85.5)</td>
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<td>Suboptimal</td>
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<td>10 (72.4)</td>
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<td>Unknown</td>
<td>19 (22.1)</td>
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<td>261 (80.6)</td>
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</table>

*Yes versus progressive disease.
*Progressive disease versus no.
*Yes versus no.
*Complete versus partial.
*Partial versus none.
*Complete versus none.
*No versus yes.
*Yes versus minimal.
*No versus minimal.
*Chemoresistant versus chemo-sensitive.
and those without recurrent disease (Table 1, footnotes b and c). A higher proportion of patients who underwent suboptimal debulking surgery showed low levels of p65 nuclear expression (24.3%, 35/144) than those who underwent optimal debulking surgery (14.5%, 24/166) or those with unknown surgical treatment (28.6%, 4/14, P = 0.04). However, we found a significant statistical correlation between cases with low and high expression of nuclear p65 and their chemoresistance and chemosensitivity (P = 0.02). Patients with high nuclear expression of p65 had longer overall survival than did patients with low p65 expression (Fig. 6A, P = 0.036). The 5-year survival time was 16 months longer in cases with high NF-κB p65 nuclear expression than in cases with low NF-κB p65 nuclear expression. Furthermore, the cumulative proportion of patients surviving 2, 5, and 10 years were greater among patients with high nuclear p65 expression than patients with low nuclear p65 expression (Supplementary Table S1). Of all the factors that influence overall disease survival, the FIGO stage, level of debulking surgery, clinical response to cisplatin-based treatment, NF-κB nuclear expression, and chemoresponse were independent prognostic factors (Supplementary Table S2). Images representing patient tumor tissues with high and low nuclear expression of p65 are shown in Figure 6B. These results suggest that NF-κB p65 nuclear activation is associated with clinical chemosensitivity and increased overall survival in patients with ovarian cancer.

Discussion

Resistance to first-line chemotherapy is the most difficult problem in the management of ovarian cancer, partly owing to our poor understanding of the underlying mechanisms of this resistance. In this report, we show that NF-κB is a biphasic regulator in ovarian cancer development; it functions not only as a tumor suppressor in ovarian cancer cells and sensitizes the cells to apoptosis induced by carboplatin and paclitaxel but also as an oncogene in more aggressive variants ovarian cancer cells and generates resistance to these therapeutic agents. NF-κB may sensitize the primary ovarian cancer cells to the initial treatment with carboplatin and paclitaxel; however, as these ovarian cancer cells in which NF-κB acts as an apoptotic sensor are eradicated, the NF-κB in the surviving cells is reprogrammed to become an oncogene to generate drug resistance and stimulate tumor growth. NF-κB inhibits antiapoptosis through enhanced MAPK phosphorylation, but it functions in an opposite way to promote antiapoptosis through dephosphorylation of MAPK in more aggressive ovarian cancer cells. We further show that NF-κB inhibits ovarian tumor growth by suppressing the expression of PP1a, which leads to the phosphorylation of MEK1/2 and ERK1/2 and the inhibition of antiapoptosis. Thus, our study provides the first experimental evidence that NF-κB can serve as a tumor suppressor through its proapoptotic function in ovarian cancer cells. However, it may also function in aggressive recurrent ovarian cancer as a tumor suppressor or as an oncogene depending on the environmental conditions.
NF-κB in Ovarian Cancer

a key regulator of resistance to platinum-based chemotherapy, the most difficult problem in the management of ovarian cancer patients following initial surgical debulking (Fig. 6C).

Our results show that NF-κB is oncogenic in 2 ovarian cancer cell lines, SKOV3.ip1 and HEYA8, which is consistent with the results of a previous study (30). The mechanism for the oncogenic constitutive activation of NF-κB in cancer has been well documented: activation of NF-κB is associated with upregulation of cell survival signaling molecules, including Bcl-2, Bcl-xL, survivin, and XIAP; cell proliferative markers, including cyclin D and c-Myc; angiogenic factors such as interleukin 8 (IL-8), VEGF, and COX-2; metastatic factors, including intercellular adhesion molecule-1, fibronectin, and CXCXR4; and inflammatory cytokines and immune responsive molecules, such as IL-6, TNF-α, MHC-I, and MHC-II (3, 12). However, emerging evidence shows that NF-κB can also function as a tumor suppressor, especially in chemically induced skin and liver cancers (8, 46). The potential tumor-suppressing mechanisms include activation of cell death signaling molecules such as Fas-associated death domain and death receptors 4 and 5 (47) and tumor suppressors including p53 and ARF (48, 49). Death receptors 4 and 5 can be activated by NF-κB through the CD437-mediated degradation of lKBo in the prostate cancer cell line DU145 (47). Nuclear localization of p53 has been reported to be associated with proapoptotic functions and transcription repression (30). Our data provide another example in an increasing list that NF-κB functions as a tumor suppressor.

Our conclusion was further confirmed by the results from our large cohort of primary clinical samples with high-grade serous carcinoma. Unlike other studies, we measured only the nuclear localization of the active form of NF-κB (phosphorylation site T435). The use of different antibodies that detect the nuclear and cytoplasmic expression of NF-κB may just reflect the total protein expression of the protein rather than the activation status (26). We have observed similar staining pattern by using several other anti-p65 antibodies; a high level of p65 nuclear localization correlates with increased chemosensitivity in patients and predicts longer overall survival.

Our data provide strong evidence that NF-κB can function as a biphasic regulator, either suppressing or enhancing ovarian cancer through the regulation of MAPK and cellular apoptosis. As NF-κB can be reprogrammed to a tumor-promoting oncogene conferring drug resistance following standard chemotherapy with carboplatin and paclitaxel, clinical treatment of ovarian cancer by using anti–NF-κB agents should be cautiously considered and guided by specific markers that can distinguish whether NF-κB is functioning as a tumor suppressor or an oncogene. The overall function of NF-κB may ultimately depend on whether it is proapoptotic or antiapoptotic in particular cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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