Inhibition of NFκB Increases the Efficacy of Cisplatin in \textit{in Vitro} and \textit{in Vivo} Ovarian Cancer Models*

Seiji Mabuchi†, Masahide Ohmichii†‡, Yukihiro Nishio‡, Tadashi Hayasaka‡, Akiko Kimura‡, Tsuyoshi Ohta§, Maki Saito§, Jun Kawagoe‡, Kazuhiro Takahashi‡, Namiko Yada-Hashimoto‡, Masahiro Sakata†, Teiichi Motoyama, Hiroshi Kurachi‡, Keiichi Tasaka‡, and Yuji Murata‡

From the †Department of Obstetrics and Gynecology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan and the Departments of ‡Obstetrics and Gynecology and §Pathology, Yamagata University, School of Medicine, 2-2-3 Iidanishi, Yamagata 990-8585, Japan

Whether or not inhibition of NFκB increases the efficacy of cisplatin in \textit{in vitro} and \textit{in vivo} ovarian cancer models was investigated. We compared the basal levels of phosphorylation of IκBα and activity of NFκB between cisplatin-sensitive A2780 cells and cisplatin-resistant Caov-3 cells. The basal levels of phosphorylation of IκBα and activity of NFκB in Caov-3 cells were significantly higher than those in A2780 cells. Cisplatin caused a more marked decrease in the phosphorylation of IκBα and activity of NFκB in A2780 cells than in Caov-3 cells. Thus, high basal levels of phosphorylation of IκBα and activation of NFκB and low marked inhibition of the phosphorylation of IκBα and activation of NFκB by cisplatin seem to reduce the sensitivity of cells to cisplatin. Inhibition of NFκB activity either by treatment with the IκBα phosphorylation inhibitor (BAY 11-7085) or a specific NFκB nuclear translocation inhibitor (SN-50) or by transfection of p50ΔNLS (which lacks the nuclear localization signal domain) increased the efficacy of both the cisplatin-induced attenuation of IκBα phosphorylation and NFκB activity and the cisplatin-induced apoptosis. In addition, treatment with BAY 11-7085 increased the efficacy of the cisplatin-induced attenuation of both the expression of x-linked inhibitor of apoptosis protein (XIAP) and cell invasion through Matrigel. Moreover, treatment with BAY 11-7085 increased the efficacy of the cisplatin-induced inhibition of the intra-abdominal dissemination and production of ascites using athymic nude mice inoculated intraperitoneally with Caov-3 cells. These results suggest that combination therapy of cisplatin with the NFκB inhibitor should increase the therapeutic efficacy of cisplatin.

The sensitivity of cells to chemotherapeutic drug-induced apoptosis appears to depend on the balance between proapoptotic and antiapoptotic signals. Therefore, it is possible that antiapoptotic signals such as the PI3K–Akt survival cascade are involved in sensitivity to chemotherapeutic drugs. We reported that Akt inactivation sensitizes human ovarian cancer cells to cisplatin (1) and paclitaxel (2), suggesting that Akt inactivation could be a hallmark for examining the sensitivity of cells to some chemotherapeutic drugs. Possible mechanisms by which Akt promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (3, 4). Akt also phosphorylates and inactivates the Forkhead transcription factors, resulting in reduced expression of the cell cycle inhibitor p27Kip1 and the Fas ligand (5–7). Via the phosphorylation of IκB kinase, Akt also activates NFκB, a transcription factor that has been implicated in cell survival (8, 9).

NFκB is activated in certain cancers and in response to chemotherapy and radiation. NFκB normally resides in the cytoplasm as an inactivated form in a complex with IκBα. Phosphorylation of IκBα by upstream kinases promotes its degradation, allowing NFκB to translocate to the nucleus and induce target genes (6, 7). The transcriptional activation of genes associated with cell proliferation (10), angiogenesis (11, 12), metastasis (13, 14), and suppression of apoptosis (15) appears to lie at the heart of the ability of NFκB to promote oncogenesis (16) and cancer therapy resistance (17, 18). While activation of NFκB may induce apoptosis in certain situations (19–22), most reports suggest that NFκB mediates survival signals that counteract apoptosis (23–28). It has been reported that genetically or constitutively activated NFκB may be critical in the development of drug resistance in cancer cells (29–31). Therefore, several agents that are able to inhibit NFκB function might be considered as an adjuvant approach in combination with chemotherapy for a variety of cancers.

These considerations led us to examine whether the status of NFκB activity is involved in sensitivity to cisplatin. In the present study, we show that the basal levels of phosphorylation of IκBα and activity of NFκB in cisplatin-resistant Caov-3 cells are significantly higher than those in cisplatin-sensitive A2780 cells. BAY 11-7085, a known pharmacological inhibitor of IκBα phosphorylation (32), enhanced the cisplatin-induced inhibition of IκBα phosphorylation and NFκB activity and increased the efficacy of cisplatin in \textit{in vitro} and \textit{in vivo} ovarian cancer models.

**EXPERIMENTAL PROCEDURES**

Materials—The anti-IκBα and phospho-IκBα antibodies were obtained from Cell Signaling Technology (Beverly, MA). The anti-NFκB TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP; i.p., intraperitoneally; NLS, nuclear localization signal.
p65 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The IκBα phosphorylation inhibitor BAY 11-7085 was purchased from Alexis Biochemicals (San Diego, CA). The specific NFκB nuclear translocation inhibitor SN-50 was purchased from Bionol (Plymouth Meeting, PA). Anti-PARP, cleaved PARP, and the X-linked inhibitor of apoptosis protein (XIAP) antibodies were obtained from Cell Signaling Technology. Anti-β-actin antibody was purchased from Sigma. ECL Western blotting detection reagents were obtained from Amersham Biosciences. The cell titer 96-well proliferation assay was obtained from Promega (Madison, WI). The terminal deoxynucleotidyltransferase- mediated dUTP nick end-labeling (TUNEL) kit (ApopTag®) was obtained from Chemicon (Temecula, CA).

Cell Cultures—Human ovarian papillary adenocarcinoma cell line Caov-3 was obtained from the American Type Culture Collection. The human ovarian cancer A2780 cell line derived from a patient prior to treatment was kindly provided by Dr. T. Tsurow (Institute of Molecular and Cellular Biosciences, Tokyo, Japan) and Drs. R. F. Ozols and T. C. Hamilton (NCI, National Institutes of Health, Bethesda, MD) (33). The cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in a water-saturated atmosphere of 95% air and 5% CO2.

 Constructs—The NFκB reporter plasmid (pElam-luc) was a kind gift from Dr. J. Cheng (University of South Florida College of Medicine) (34). pCR-FLAG-p50 (encoding full-length human p50 with the FLAG epitope) and pCR-FLAG-p50ΔNLS (encoding nuclear localization signal (NLS)–deleted p50 with the FLAG epitope) constructs were kind gifts from Dr. Gourisankar Ghosh (University of California, San Diego) Chen et al. (39).

 Proliferation Assay—Cell proliferation (35) was assessed by the addition of cisplatin at the indicated concentrations for 48 h, 1 day after seeding test cells into 96-well plates. The number of surviving cells was determined 24 h later by determination of the number of surviving cells was determined using the Bio-Rad protein assay and then measured using the Cell-Titer 96-well proliferation assay (Promega). The mean of the duplicate wells was calculated and was expressed as a percentage of the confluent control.

 RESULTS

 Differences of IκBα Phosphorylation and NFκB Activity Depending on the Sensitivity to Cisplatin—The sensitivity to cisplatin of A2780 and Caov-3 cells was examined using the MTS assay (Fig. 1A). It was first confirmed thereby that A2780 cells are sensitive and Caov-3 cells are resistant to cisplatin, as reported previously (1, 35). Since constitutive phosphorylation of IκBα together with increased NFκB activity has been reported to reduce sensitivity to chemotherapeutic drugs and is associated with invasive behavior of cancer cells (38), we next compared the IκBα phosphorylation (Fig. 1B) and NFκB activity (Fig. 1C) between cisplatin-sensitive A2780 cells and cisplatin-resistant Caov-3 cells. Cells were treated with 200 μM cisplatin for 30 min and used to prepare lysates that were analyzed by Western blotting with anti-phospho-IκBα and anti-NFκB p65 antibodies. The basal levels of phosphorylation of IκBα were significantly higher than that in A2780 cells (Fig. 1B, panels i and ii). Although cisplatin caused a decrease in the level of phosphorylated IκBα in both Caov-3 and A2780 cells, the degree of the decrement of phosphorylated IκBα by cisplatin in A2780 cells was more marked than that in Caov-3 cells (Fig. 1B, panels i and ii). The levels of IκBα did not differ among the lines (Fig. 1B, panel iii), suggesting that cisplatin had no effect on the degradation of IκBα in either Caov-3 or A2780 cells. To assess the NFκB activity, cells were transfected with a NFκB-luciferase reporter plasmid. The basal NFκB activity in Caov-3 cells was significantly higher than that in A2780 cells (Fig. 1B, panels i and ii). Although cisplatin caused a decrease in the level of phosphorylated IκBα in both Caov-3 and A2780 cells, the degree of the decrement of phosphorylated IκBα by cisplatin in A2780 cells was more marked than that in Caov-3 cells (Fig. 1B, panels i and ii). Total levels of IκBα did not differ among the lines (Fig. 1B, panel iii), suggesting that cisplatin caused no effect on the degradation of IκBα in either Caov-3 or A2780 cells. To assess the NFκB activity, cells were transfected with a NFκB-luciferase reporter plasmid. The basal NFκB activity in Caov-3 cells was significantly higher than that in A2780 cells (Fig. 1C). Although cisplatin caused a decrease in NFκB activity in both Caov-3 and A2780 cells, the degree of the decrement of NFκB activity caused by cisplatin in A2780 cells was more marked than that in Caov-3 cells (Fig. 1C). These results suggest that both high basal levels of phosphorylation of IκBα and activation of NFκB and weaker decrements of the phosphorylation of IκBα and activation of NFκB by cisplatin seem to reduce the sensitivity of cells to cisplatin.
Effect of Inhibition of NFκB Activity on Cisplatin-induced Apoptosis—We next examined whether inhibition of NFκB activity increased the efficacy with which cisplatin induced the attenuation of IκB phosphorylation and NFκB activity and also induced apoptosis. We confirmed that treatment with an IκB phosphorylation inhibitor (BAY 11-7085) (32) attenuated both the phosphorylation of IκBα (Fig. 2A, ii, panels a and b) and NFκB activity (Fig. 2B, panel i), and a specific NFκB nuclear translocation inhibitor (SN-50) attenuated the NFκB activity (Fig. 2B, panel ii). In addition, treatment with BAY 11-7085 increased the efficacy with which cisplatin induced the attenuation of IκB phosphorylation (Fig. 2A, iii, panels a and b) and NFκB activity (Fig. 2B, panel iii), whereas treatment with SN-50 increased the efficacy with which cisplatin induced the attenuation of NFκB activity (Fig. 2B, panel iii). Total levels of IκBα did not differ among the lanes (Fig. 2A, panel c), like the basal expression of β-actin (Fig. 2A, panel d), suggesting that cisplatin and/or BAY 11-7085 had no effect on the degradation of IκBα. Furthermore, we examined the effect of another NFκB inhibitor on the cisplatin-induced attenuation of NFκB activity. The best characterized species of NFκB dimer is the p50/p65 heterodimer (39). A previous report demonstrated that the NLS poly peptide (p50) is required for the translocation of the dimer to the nucleus (40) and that p50ΔNLS lacking the NLS domain inhibits the nucleocytoplasmic shuttling of NFκB dimers. Therefore, we examined the effect of p50ΔNLS on the cisplatin-induced attenuation of NFκB activity (Fig. 2B, panel iv). Transfection of p50ΔNLS significantly increased the efficacy with which cisplatin induced the attenuation of NFκB activity compared with the effects in cells expressing wild-type p50 (Fig. 2B, panel iv). Thus, these data indicated that NFκB inhibitors increased the efficacy with which cisplatin induced the attenuation of NFκB activity.

In the presence of BAY 11-7085 or SN-50, the ability of cisplatin to induce growth inhibition was significantly enhanced (Fig. 2C). Moreover, we examined the effects of BAY 11-7085 (Fig. 2D, panel i) and SN-50 (Fig. 2D, panel ii) on the cisplatin-induced cleavage of PARP by Western blotting with anti-PARP antibody (Fig. 2D, panel b), anti-cleaved PARP antibody (Fig. 2D, panel c), or anti-β-actin antibody (Fig. 2D, panel d). Cisplatin induced the cleavage of PARP (Fig. 2D, panels a, b, and c). BAY 11-7085 (Fig. 2D, i, panels a, b, and c) and SN-50 (Fig. 2D, ii, panels a, b, and c) significantly enhanced the ability of cisplatin to induce the cleavage of PARP,
whereas the expression of β-actin was not changed by any of these treatments (Fig. 2D, panel d).

Effect of Inhibition of NFκB Activity on the Cisplatin-induced Attenuation of the Expression of Survival Genes—NFκB regulates the expression of a number of antiapoptotic genes (41–43). Among them are the family of inhibitor of apoptosis proteins (IAPs), which play a central role in repressing caspase-mediated cell death. It was reported that cisplatin inhibits the expression of XIAP (44) and that down-regulation of XIAP induces apoptosis and increases cisplatin sensitivity (45), suggesting that XIAP is a determinant of cisplatin sensitivity in ovarian cancer. Therefore, we examined the effect of cisplatin and BAY 11-7085 alone and in combination on the expression of XIAP. Although the expression of β-actin did not vary among the lanes, both cisplatin and BAY 11-7085 partially attenuated the expression of XIAP, and co-treatment with cisplatin plus BAY 11-7085 completely inhibited the expression of XIAP (Fig. 3).

Effect of Inhibition of NFκB Activity on the Cisplatin-induced Attenuation of Invasion of Caov-3 Cells through Matrigel—Because it was reported that NFκB is involved in invasiveness and metastatic properties (46), we examined the effects of cisplatin and BAY 11-7085 alone and in combination on the invasion of Caov-3 cells through Matrigel. Under laboratory conditions, Caov-3 cells migrated through Matrigel. Whereas either cisplatin or BAY 11-7085 partially inhibited cell invasion through Matrigel, co-treatment with cisplatin plus BAY 11-7085 completely inhibited cell invasion through Matrigel (Fig. 4).
Effect of BAY 11-7085 on the Cisplatin-induced Inhibition of Intra-abdominal Dissemination of Ovarian Cancer

Ovarian cancer is clinically silent and half of the patients are first detected at an advanced stage with ascites and peritoneal dissemination (47). Because peritoneal dissemination is the main process of progression in ovarian cancer and the amount of ascitic fluid and the size of the disseminated tumor are correlated with the patient’s prognosis (48), controlling ascitic fluid and peritoneal dissemination are crucial in ovarian cancer therapy. We therefore examined the effect of cisplatin and BAY 11-7085 alone and in combination on the control of intra-abdominal dissemination of ovarian cancer and ascites formation to assess whether combination therapy would increase the therapeutic efficacy of each agent. Athymic nude mice were inoculated (i.p.) with Caov-3 cells or growth medium. Two weeks after inoculation, athymic nude mice inoculated (i.p.) with Caov-3 cells were randomized into four groups treated with the following for 4 weeks: (a) vehicle (PBS); (b) cisplatin (5 mg/kg) once a week; (c) BAY 11-7085 (5 mg/kg) once a week; and (d) cisplatin (5 mg/kg) once a week + BAY 11-7085 (5 mg/kg) three times a week. The appearance of ascites was shown in Fig. 5A. The volume of ascites was measured at autopsy (Fig. 5B). The volume of ascites was significantly higher in athymic nude mice inoculated (i.p.) with Caov-3 cells than in athymic nude mice inoculated (i.p.) with growth medium. Pathological examination was performed to determine...
the extent of intra-abdominal dissemination at autopsy. Intra-abdominal dissemination was clearly detected in athymic nude mice inoculated (i.p.) with Caov-3 cells followed by treatment with vehicle (Fig. 6, panel a, and B), consistent with Caov-3 cells. Cisplatin alone (Fig. 5A, panel b) or BAY 11-7085 alone (Fig. 5A, panel c) significantly diminished the volume of ascites compared with vehicle control. The combination of cisplatin + BAY 11-7085 (Fig. 5B, panel d) further enhanced the inhibitory effects on the production of ascites. Cisplatin alone (Fig. 6A, panel b) or BAY 11-7085 alone (Fig. 6A, panel c) also apparently diminished the extent of intra-abdominal dissemination. The combination of cisplatin + BAY 11-7085 (Fig. 6A, panel d) further enhanced each inhibitory effect on intra-abdominal dissemination. These results suggest that combination therapy of cisplatin with BAY 11-7085 would increase the therapeutic efficacy of cisplatin.

Intra-abdominally disseminated tumors harvested from the different groups were processed for immunohistochemical analyses. Apoptosis was analyzed by the TUNEL method (Fig. 7). The fraction of TUNEL-positive cells was higher in tumors from mice treated with cisplatin + BAY 11-7085 than in tumors from mice treated with vehicle alone (Fig. 7A). Apoptosis was quantitated in tumors of mice treated with vehicle alone (panel a), cisplatin alone (panel b), BAY 11-7085 alone (panel c), and cisplatin + BAY 11-7085 (panel d) (Fig. 7B) as described for the experiment shown in Fig. 5. Cisplatin alone (Fig. 7B, panel b) or BAY 11-7085 alone (Fig. 7B, panel c) significantly increased the proportion of apoptotic cells in tumors compared with vehicle alone (Fig. 7B, panel a). The combination of cisplatin + BAY 11-7085 (Fig. 7B, panel d) further enhanced the increase of the proportion of apoptotic cells in tumors. We further confirmed whether BAY 11-7085 blocked the NFκB cascade in vivo. IκBα phosphorylation status and subcellular localization of NFκB p65 were assessed by immunohistochem-
Specificity of NFκB to determine the chemotherapy specificity and potential tumor for p53-dependent cell death (50). More studies will be needed.

We reported that Akt inactivation and inhibition of BAD phosphorylation sensitize human ovarian cancer cells to cisplatin. Although NFκB is a substrate of Akt (like BAD and Forkhead), NFκB activation is involved in angiogenesis (11, 12) and metastasis (13, 14) in addition to the suppression of apoptosis. Therefore, NFκB inhibitors might increase the efficacy of chemotherapies in both primary and metastatic lesions. It was reported that NFκB inhibitors induce adhesion-dependent colon cancer apoptosis (52). We showed in this study that treatment of athymic mice with BAY 11-7085 enhanced the ability of cisplatin to inhibit tumor implantation into the liver and peritoneum (Fig. 6A). In addition, BAY 11-7085 increased the ability of cisplatin to inhibit both cell proliferation in an MTS assay (Fig. 2C) and cellular invasion in an in vitro invasion assay (Fig. 4). Thus, NFκB inhibitors might increase the efficiency with which cisplatin inhibits both primary and metastatic lesions. Glycogen synthase kinase-3α (53) and endothelial nitric-oxide synthase (54, 55) are also other Akt substrates, and Akt is thus also involved in metabolic processes and vessel dilatation, respectively. Therefore, it is possible that inhibition of PI3K/Akt activation is not a safe strategy for preventing chemoresistance. Accordingly, NFκB inhibitors might be more useful for sensitization to chemotherapeutic drugs than agents that are able to inhibit PI3K/Akt activity.

Constitutive activation of NFκB has been described in a great number of solid tumors, and this activation appears to support cancer cell survival and to reduce the sensitivity to chemotherapeutic drugs. We showed in this study that whereas cisplatin-sensitive A2780 cells do not have constitutive activation of NFκB, cisplatin-resistant Caov-3 cells do have constitutive activation of NFκB. Thus, it appears that constitutive activation of NFκB mediates cisplatin resistance in ovarian cancer cells and inhibition of NFκB activation sensitizes the ovarian cancer cells to cisplatin.

How do NFκB inhibitors cause the inhibition of growth of human ovarian cancer cells? It was reported that NFκB inhibitors diminished the expression of survival genes regulated by NFκB, such as c-IAP-2, TRAF-1, TRAF-2, XIAP, or IEX-1L (42, 43). We also showed that NFκB inhibitors caused the inhibition of survival genes regulated by NFκB, such as c-IAP-2, TRAF-1, TRAF-2, XIAP, or IEX-1L (42, 43)
of the expression of survival genes in human ovarian cancer cells (Fig. 3). The fact that NFκB mediates the expression of multiple survival genes makes it an important and rational target for cancer chemotherapy.

BAY 11-7082 is also a known pharmacological inhibitor of IκBα phosphorylation (32), like BAY 11-7085. In the presence of BAY 11-7082, the cisplatin-induced attenuation of IκBα phosphorylation was significantly enhanced (data not shown). BAY 11-7082 and BAY 11-7085 also activate the c-Jun N-terminal protein kinase and p38 (32), both of which are known to be involved in the induction of apoptosis (56).

Thus, the effects of BAY 11-7082 and BAY 11-7085 do not exclude the role of cellular proteins other than NFκB. Five homologous polypeptides, p50, p65, c-Rel, RelB, and p52, comprise the mammalian Rel/NFκB transcription factor family. The subunits associate in a combinatorial fashion to form transcriptionally active homo- and heterodimers. The best characterized species of NFκB dimer is the p50/p65 heterodimer (39). A previous report demonstrated that the NLS polypeptide of p50 is required for its translocation to the nucleus (40) and that p50ΔNLS lacking the NLS domain inhibits the nucleocytoplasmic shuttling of NFκB dimers. Therefore, we examined the effect of p50ΔNLS on the attenuation of IκBα phosphorylation by cisplatin. Transfection of p50ΔNLS significantly inhibited the increased efficacy of the cisplatin-induced attenuation of NFκB activity compared with the effect in cells expressing wild-type p50 (Fig. 2B, panel iv). Thus, the similarity of the effects caused by treatment with BAY 11-7082 or BAY 11-7085 and by transfection with p50ΔNLS suggests that inhibition of NFκB activity has strong potential as a novel adjuvant chemotherapy.

Most studies of the inhibition of NFκB activity in vivo have used a gene therapy approach through the introduction of a nondegradable IκB mutant that prevents nuclear translocation of NFκB. The advantage of soluble inhibitors is that their delivery would be easier and more efficient than gene transfer in vivo. We did not detect significant renal, hepatic, or pulmonary tissue toxicity in this study. A previous study also showed that using up to 20 mg/kg/day of these agents in rats for 21 days did not cause obvious toxicity (32).

Activation of NFκB via phosphorylation of an inhibitor protein (IκB) leads to degradation of IκB through the ubiquitin-proteasome pathway. Inhibition of IκB degradation by proteasome inhibitors keeps NFκB in the cytoplasm, thereby preventing it from acting on nuclear DNA (57, 58). PS-341, which is a potent, boronic acid dipeptide that is highly selective for proteasome inhibition, can be systemically administered clinically (59). PS-341 has been shown to enhance the apoptotic response to chemotherapy in a variety of in vitro and in vivo models (18, 60–62). A Phase I trial of PS-341 and carboplatin in recurrent ovarian cancer is currently ongoing (51). A Phase II trial of PS-341 for the treatment of recurrent platinum-sensitive ovarian or primary or peritoneal cancer (GOG 146-N) is also being conducted. We now await the results of these currently ongoing clinical trials.

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