Abstract. Paclitaxel is used frequently for the treatment of patients with non-small cell lung cancer. Hypersensitivity reactions remain one of the major adverse events in the clinical use of paclitaxel. Glucocorticoids are used to prevent these adverse events. This study was carried out in order to clarify the effect of glucocorticoids on paclitaxel-induced cytotoxity of cancer cells. Pretreatment with 10 μM of dexamethasone inhibited ERK activation and subsequent retinoblastoma protein (pRB) phosphorylation, and reduced sensitivity to paclitaxel in A549 cells. Then, we utilized ERK (PD98059) and AKT (LY294002) inhibitors. PD98059 and LY294002 effectively suppressed pRB phosphorylation in A549 cells. Dexamethasone (10 μM) suppressed ERK activity as well as PD98059, although it did not affect AKT activity. Furthermore, the combinations of paclitaxel with PD98059 or LY294002 were similarly antagonistic. Our observation in this study raised the possibility that dexamethasone pretreatment antagonizes paclitaxel-induced cytotoxicity through ERK suppression and pRB dephosphorylation. These observations support the development of new generation taxane-based chemotherapy without glucocorticoid premedication.

Introduction

Lung cancer is now one of the most common causes of cancer-related death throughout the world (1,2). Although platinum-based chemotherapy remains the cornerstone of treatment for the NSCLC subtype, it results in a small improvement in survival compared with supportive care alone (1,3). Recently, newer chemotherapeutic agents such as taxanes, vinorelbine, gemcitabine and irinotecan have become clinically available to NSCLC.

Among these newer drugs, paclitaxel is the one used frequently for the treatment of patients with NSCLC. The mechanism of cell death induced by paclitaxel is not entirely clear, but it is widely thought that the major cellular target for paclitaxel is the tubulin/microtubule system in mitosis (4). In the clinical use of paclitaxel, hypersensitivity reactions are one of the major adverse events, and glucocorticoids, including dexamethasone, are used to prevent these side-effects (5-7). Because glucocorticoid premedication is routinely used in treatment with paclitaxel, it is important to evaluate its effect on the paclitaxel-induced cytotoxicity of cancer cells to improve the effectiveness of paclitaxel-based chemotherapy.

Glucocorticoids are regulatory molecules that control metabolism, development, inflammation, cell growth, proliferation, and differentiation (8). Furthermore, glucocorticoids are known to exert antiproliferative effects in a number of different tissues and cell types resulting in G1 cell cycle arrest and/or apoptosis (8-13).

Retinoblastoma protein (pRB) is a key regulator of cell cycle progression (14,15), and its phosphorylation is essential for S-phase entry from G1 phase in the cell cycle (16). In the previous study, we reported that pRB phosphorylation status and the cell cycle progression during paclitaxel treatment are important determinants of sensitivity to paclitaxel in NSCLC cells (17). Since glucocorticoid itself affects proliferation of cancer cells (8-13), drug interaction between paclitaxel and glucocorticoid may be possible through the modification of pRB phosphorylation status by glucocorticoid.

Accordingly, we hypothesized that pretreatment of NSCLC cells with dexamethasone may affect the sensitivity to paclitaxel. To address this issue, we investigated the effects of dexamethasone pretreatment on the paclitaxel-induced cytotoxicity in relation to pRB phosphorylation status and its upstream regulators using A549 lung adenocarcinoma cells.

Materials and methods

Chemicals and reagents. Paclitaxel (a gift from Bristol-Myers-Squibb, Inc., Tokyo, Japan); 2'-Amino-3'-methoxyflavone (PD98059) (Calbiochem, San Diego, CA, USA) and 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Sigma-Aldrich Japan, Tokyo, Japan), was dissolved in dimethylsulfoxide and stored at -20°C. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
(Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) and stored at -20°C.

Cells. The human NSCLC cell line, A549 cells, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells were grown in a humidified atmosphere of 5% CO₂-95% air.

Cell lysis, immunoprecipitation, and Western blot analysis. Cells were lysed in a modified radioimmune precipitation buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 40 mM β-glycerophosphate, and 2 mM Na₃VO₄), and insoluble material was removed by centrifugation. Protein concentration was determined by means of a Bio-Rad Protein assay (Bio-Rad, CA) and lysates containing 50 μg of total cellular protein or immunoprecipitates with indicated antibodies were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech) using goat anti-rabbit IgGs coupled to horseradish peroxidase as a secondary antibody (Amersham Pharmacia Biotech). Antibody against pRb (IF8) was purchased from Santa Cruz Biotechnology. Anti-phosphorylated pRB (Ser 795) and anti-pRB C-terminal antibodies were purchased from Cell Signaling Technology. Anti-ERK1/2 and anti-active ERK antibodies were purchased from Promega Corporation. Anti-AKT and anti-active AKT antibodies were purchased from Cell Signaling Technology.

Cell cycle analysis. A549 cells were incubated for 24 h in 1 ml of DMEM containing 10% FCS using 6-well flat bottom multiplates (Sumitomo Bakelite Co., Tokyo, Japan). After 24 h, growth media was removed. Cells were pretreated with or without 10 μM of dexamethasone for 12 h. After dexamethasone containing growth media was removed, 5 nM of paclitaxel was added to each well. Cells were incubated for 48 h further. The cells were washed, collected by trypsinization and resuspended in 1 ml phosphate-buffered saline (PBS). The cells were fixed in ice-cold 70% ethanol overnight at 4°C and resuspended in 0.5 ml of PBS containing RNase A (1 μg/ml). Cell fluorescence was analyzed on a Becton FACScan, using cell Quest software (Becton Dickinson, Mountain View, CA).

MTT assay. The effects of dexamethasone on the cell proliferation in A549 cells were measured by MTT assay. Cells were counted with a hematocytometer, 1x10⁶ cells were incubated in 100 μl medium for 24 h using 96-well flat bottom multiplates (Nalge Nunc International K.K., Denmark). After 24 h, cells were treated with dexamethasone at 0-100 μM for 24 and 48 h. Then, 20 μg of MTT in 10 μl PBS was added to each well and incubation was performed for an additional 4 h. Thereafter, 100 μl of 0.04 N HCl in 2-propanol was added and incubated overnight in order to solubilize the MTT formazan crystal. The absorbance of each well was measured at a 570-nm wavelength (reference 650 nm) using a scanning multi-well spectrophotometer (MPR A4i, Tosoh Co., Tokyo, Japan).

The cytotoxic activity of paclitaxel was measured by MTT assay as described above. A549 cells were exposed to various concentrations of paclitaxel in the presence or absence of pretreatment with dexamethasone for 12 h or concurrent treatment with 50 μM of PD98059 and 25 μM of LY294002.

Results

Effect of dexamethasone on proliferation in A549 cells. To investigate whether dexamethasone effects proliferation, A549 cells were treated with 0-100 μM dexamethasone for 24 and 48 h. Although the proliferation of A549 cells did not differ with dexamethasone treatment for 24 h (data not shown), cell proliferation was inhibited clearly at dexamethasone concentrations >1.0 μM after 48-h treatment (Fig. 1). Since 10 μM of dexamethasone exerted maximum growth-inhibitory effects on A549, this concentration was adopted for the following experiments.

Effects of dexamethasone pretreatment on paclitaxel-induced cytotoxic activity in A549 cells. Then, we investigated the effect of dexamethasone pretreatment on paclitaxel-induced cytotoxicity. A549 cells were pretreated with or without 10 μM dexamethasone for 12 h and exposed to various concentrations of paclitaxel for 72 h. The cytotoxic effects were evaluated by MTT assay. As shown, pretreatment with 10 μM of dexamethasone clearly inhibited the cytotoxic activity of paclitaxel (Fig. 2).

Effects of dexamethasone and paclitaxel on cell cycle distribution in A549 cells. For the purpose of linking the effect of cell cycle distribution and the biological response to dexamethasone and paclitaxel, we performed DNA content analysis by propidium-iodine staining in A549 cells. A549 cells were treated with 10 μM of dexamethasone for 12 h, 5 nM of paclitaxel for 48 h or their combination. DNA ploidy analysis by flow cytometry is shown in Fig. 3. G1-phase cells increased slightly after treatment with dexamethasone for 12 h compared with that of the control (Fig. 3A and B). Even after 48-h treatment with dexamethasone, sub-G1 cell populations did not increase (data not shown). After paclitaxel treatment for 48 h, G1-phase cells disappeared, and sub-G1 cell...
populations increased significantly, indicating apoptosis by paclitaxel (Fig. 3C). However, combination of paclitaxel with dexamethasone pretreatment for 12 h completely inhibited paclitaxel-induced apoptosis as manifested by a reduced number of cells in the sub-G1 population of A549 cells (Fig. 3D). A significant proportion of the remaining cells were still in the G1 phase, indicating the inhibition of cell cycle progression induced by the dexamethasone pretreatment.

Effects of dexamethasone and paclitaxel on pRB phosphorylation status in A549 cells. We investigated the effect of dexamethasone and paclitaxel on pRB phosphorylation. Since treatment with 5 nM of paclitaxel strongly affected the pRB phosphorylation status of NSCLC cells in our previous study (17), this concentration was used in the present study. Although treatment with paclitaxel did not change the phosphorylated status of pRB in A549 cells, treatment with 10 μM of dexamethasone for 12 h completely blocked pRB phosphorylation and it was not altered with 5 nM of paclitaxel (Fig. 4). These data indicate that pretreatment with dexamethasone for 12 h before paclitaxel treatment dephosphorylates pRB and then inhibits paclitaxel-induced cytotoxicity in A549 cells.

Effects of ERK and AKT inhibitor on the phosphorylation of pRB, and effects of dexamethasone on the activity of ERK and AKT in A549 cells. To explore the role of ERK and AKT in the dexamethasone-induced dephosphorylation of pRB, the effect of specific inhibitor of ERK, PD98059, or AKT, LY294002 was evaluated in A549 cells. Treatment with 50 μM of PD98059 and 25 μM of LY294002 suppressed the phosphorylation of pRB, indicating that, in A549 cells, ERK and AKT activation is essential for pRB phosphorylation. Then, using a specific antibody to detect the active form of ERK and AKT, we evaluated the effects of dexamethasone on the activity of these kinases in A549 cells. In A549 cells, the level of phosphorylated ERK and AKT did not change in response to paclitaxel, and this ERK and AKT activation was blocked in the presence of 50 μM of PD98059 and 25 μM of LY294002, respectively. The dexamethasone treatment inhibited ERK phosphorylation to a similar level as did PD98059 treatment (Fig. 5B). On the other hand, the level of phosphorylated AKT did not change after dexamethasone treatment (Fig. 5C).

Effects of ERK and AKT inhibitor on paclitaxel-induced cytotoxicity in A549 cells. To confirm the importance of pRB dephosphorylation in the antagonistic effect of dexamethasone on paclitaxel-induced cytotoxicity, we assessed the effect of PD98059 and LY294002 on paclitaxel-induced cytotoxicity. A549 cells were treated with paclitaxel for 72 h in the presence or absence of 50 μM PD98059 or 25 μM LY294002. The cytotoxic effects were evaluated by MTT assay. As shown, 50 μM PD98059 and 25 μM LY294002, which were sufficient to block pRB phosphorylation, clearly inhibited paclitaxel-induced cytotoxicity in A549 cells (Fig. 6).

Discussion

This in vitro study was carried out in order to clarify the effect of glucocorticoid premedication on paclitaxel-induced cytotoxicity to NSCLC cells and the underlying mechanism
by which glucocorticoid interferes with the action of paclitaxel. Our observations in the present study suggest that the ERK, but not AKT, inhibition and the subsequent dephosphorylation of pRB by pretreatment with dexamethasone are important mechanisms of its antagonistic effect on paclitaxel-induced cytotoxicity.

Using A549 cells as a model system of NSCLC, we examined the effects of dexamethasone on cell proliferation, phosphorylation status of pRB and paclitaxel-induced cytotoxicity. Treatment with dexamethasone suppressed the growth of A549 cells. Judging from the results from MTT assay and flow cytometric analysis, this growth suppression by dexamethasone is attributable to cytostatic but not cytotoxic effects. The dephosphorylation of pRB by dexamethasone can be regarded as a main mechanism of the growth inhibitory effect since its phosphorylation is essential for S-phase entry from G1 phase in the cell cycle (16).

Previously, we reported that paclitaxel activates ERK and subsequently phosphorylates pRB in a cell-specific manner, and proposed that pRB phosphorylation by paclitaxel may enhance the sensitivity to it (17). Although paclitaxel itself did not affect pRB phosphorylation in A549 cells, the proliferation-inhibitory effect and pRB dephosphorylation by dexamethasone promoted us to speculate that the combination of paclitaxel with dexamethasone may be antagonistic.

After the initial report of hypersensitive reactions of paclitaxel to the National Cancer Institute, it was recommended for all patients receiving paclitaxel to be given prophylactic therapy including dexamethasone. Since then, dexamethasone was administered 12-14 h before paclitaxel administration. So we used dexamethasone 12 h before treatment with paclitaxel (5). To approximate clinical application, we exposed cells to dexamethasone for 12 h before paclitaxel treatment. Pretreatment of A549 cells with dexamethasone suppressed the effect of paclitaxel. Judging from the decrease of sub-G1 population in dexamethasone-pretreated cells compared with those with paclitaxel alone, dexamethasone was thought to inhibit paclitaxel-induced apoptosis. In general, paclitaxel acts on cells in the M phase and induces apoptosis. Accordingly, since a major cell fraction were still in the G1 phase even after paclitaxel treatment in dexamethasone-pretreated cells, it is natural to speculate that G1-arrest induced by pRB dephosphorylation may be an important mechanism by which dexamethasone suppressed the effect of paclitaxel.

To confirm this speculation, we utilized ERK (PD98059) and AKT (LY294002) inhibitors. ERK activation potentiates the cyclin D expression and inactivates p27\(^{kip1}\) cell cycle inhibitor protein (18,19). AKT suppresses p21\(^{WAF1/CIP1}\) and p27\(^{kip1}\) function through phosphorylation (20,21), leading to pRB phosphorylation. Thus, the suppression of these two kinases should lead to the dephosphorylation of pRB. As expected, PD98059 and LY294002 effectively suppressed pRB phosphorylation in A549 cells. Dexamethasone (10 \(\mu M\)) suppressed ERK activity as well as 50 \(\mu M\) PD98059, although it did not affect AKT activity. Furthermore, the combinations of paclitaxel with PD98059 or LY294002 were similarly antagonistic. These observations are in accordance with our previous study that proposed that pRB dephosphorylation confers paclitaxel resistance to cancer cells (17). We concluded that, in A549 cells, the antagonistic effect of dexamethasone...
pretreatment on paclitaxel-induced cytotoxicity is attributable to the ERK suppression and subsequent pRB dephosphorylation.

Several previous studies reported that inhibition of ERK and AKT pathway enhanced paclitaxel-induced cytotoxicity (22-26). However, these observations are not necessarily general. Bacus et al observed that when PD98059 and paclitaxel were applied simultaneously, antagonistic effects were observed (27), in accordance with our result obtained in A549 cells. Mitsuuchi et al reported that PI3K/AKT signaling did not alter the paclitaxel-induced cytotoxicity, suggesting cell death induced by paclitaxel proceeds independently of cell protective effects of this pathway (21). Both ERK and AKT pathway mediate cell growth-promoting and anti-apoptotic signaling (28-30). Many cancer cells have abnormality of cell cycle regulators including pRB, CDKs, and CDK inhibitors (31), and deviate from the normal regulation of cell cycle. In that case, the suppression of ERK and AKT may enhance paclitaxel-induced apoptosis without affecting the cell cycle through the inhibition of the anti-apoptotic effect which is another function of ERK and AKT. On the other hand, in the case that the suppression of ERK and AKT leads to pRB dephosphorylation and G1-arrest, it is possible that inhibition of these kinases antagonizes paclitaxel-induced cytotoxicity through the decrease of paclitaxel-sensitive M phase cell fraction during paclitaxel exposure. The present study raised the possibility that dexamethasone pretreatment antagonizes paclitaxel-induced cytotoxicity, at least, in a fraction of cancer cells through ERK suppression and subsequent pRB dephosphorylation.

A new analogue and formulation of paclitaxel are being developed (32,33), leading to the decrease or removal of polyoxylatedcastor oil that causes hypersensitivity reactions in the use of conventional paclitaxel preparation. Even if these drugs have the same activity against cancer cells as paclitaxel, it may be possible that the omission of glucocorticoid pretreatment will lead to higher effectiveness. Our observation in this study supports this possibility and promotes the development of chemotherapies using a new generation of taxane that obviates glucocorticoid predication.

In conclusion, the present study demonstrated that pretreatment with dexamethasone before paclitaxel exposure antagonized paclitaxel-induced cytotoxicity through inhibition of ERK and pRB phosphorylation in A549 cells. These observations support the development of new generation taxane-based chemotherapy without glucocorticoid predication.

References