CDK4 Down-Regulation Induced by Paclitaxel Is Associated with G1 Arrest in Gastric Cancer Cells

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ABSTRACT
Paclitaxel induces a cell cycle block at G2-M phase by preventing the depolymerization of microtubules and induces p53-independent apoptosis in many cancer cells. We observed that gastric cancer cells treated with paclitaxel have shown a cyclin-dependent kinase (CDK4) down-regulation. This paclitaxel-induced CDK4 down-regulation resulted in a cell cycle arrest at G1-S phase. To confirm this observation, we prepared stable transfectants that overexpressed CDK4 and analyzed the cell cycle progression. Ectopic expression of CDK4 in SNU cells resulted in a release of paclitaxel-induced G1 arrest. The release of G1 arrest by enforced expression of CDK4 seems to make the cells more sensitive to paclitaxel-induced apoptosis. From this finding, we could then suggest that paclitaxel treatment induces both G1-S and G2-M blocks in the cell cycle progression of gastric cancer cells.

INTRODUCTION
Paclitaxel is cytotoxic to many kinds of cancer cells including ovarian cancer, astrocytoma, prostate cancer, and gastric cancer in vitro (1–5). It is used as one of the most effective chemotherapeutic drugs for ovarian and breast cancer (6). Paclitaxel is also effective for various other malignant tumors, including lung, head and neck, malignant melanoma, and so forth (7–10). However, the clinical activity of paclitaxel in gastric cancer was uncertain. Although the Eastern Cooperative Oncology Group has demonstrated that paclitaxel has minimal activity in gastric cancers (11), paclitaxel has shown activity against gastric cancer cell lines in vitro (1). Investigators from the M. D. Anderson Cancer Center (Houston, TX) and Memorial Sloan-Kettering Cancer Center (New York, NY) had obtained a response rate of 36% when using paclitaxel against adenocarcinomas of the distal esophagus (12); the response rate is similar or identical with proximal gastric cancer (13). In a Phase II study of paclitaxel against advanced gastric cancer, the 24-h infusion of paclitaxel resulted in a 23% response rate at a dosage of 200 mg/m2, demonstrating a significant role for paclitaxel in gastric cancer treatment (14).

Paclitaxel treatment arrests the cell cycle at G2-M phase by preventing the depolymerization of microtubules and this G2-M block results in an apoptotic cell death (15–19). Studies on the correlation between cell cycle and paclitaxel sensitivity demonstrated that the cytotoxicity induced by paclitaxel was maximal at G2-M phase whereas it was minimal at G1-S phase (19). Another report showed that a low concentration of paclitaxel was cytotoxic to gastric cancer cells at G0-G1 phase (1). The cell cycle was arrested at G1 phase in the presence of higher doses of paclitaxel. These results suggested that the cytotoxic effect of paclitaxel varied with drug concentration. In this study, we observed that with paclitaxel treatment, gastric cancer cells were arrested not only at G2-M phase but also at G1-S phases. The cell cycle arrest at G1-S phase correlated with CDK4 down-regulation caused by paclitaxel.

MATERIALS AND METHODS
Cell Culture and Reagents. AGS and Korean gastric cancer cell lines (20) SNU1, SNU5, SUN16, SUN601, SUN638, and SNU668 were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 7% fetal bovine serum (Life Technologies, Inc.), 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin and were maintained at 37°C and 5% CO2. Paclitaxel was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO. Enhanced chemiluminescent Western blot detection reagents were purchased from Amersharm (Arlington Heights, IL). Anti-CDK4 antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Construction of CDK4-overexpressing Cell Lines. Exponentially growing SNU638 cells (5 × 105) were transferred to a 60-mm tissue culture dish containing 4 ml of RPMI 1640. After being washed with serum-free medium, cells were incubated with a mixture of 20 μl of Lipofectin (Life Technologies, Inc.) and 4 μg of the CDK4 DNA in 2 ml of serum-free medium for 12 h. The cells were then cultured in RPMI 1640 containing 500 μg/ml G418 (Life Technologies, Inc.) for 2–3 weeks until individual colonies were formed on plates. Ten colonies were isolated and cultured for the use of Western blotting.

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The abbreviation used is: CDK, cyclin-dependent kinase.
Western Blotting. Cells (2 x 10^6) were treated with various concentrations of paclitaxel for 24 h and cell lysates were prepared by lysis using 50 μl of RIPA buffer (150 mM NaCl, 1.0% NP40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)) containing phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM NaF, 1 mM phenyl methylsulfonyl fluoride, and 30 mM NaPPi). Equal amounts of protein (50 μg/ml) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Filters were probed with anti-CDK4 antibody, which was developed with peroxidase-labeled antirabbit antibody according to the manufacturer's instructions (Amersham, Little Chalfont, England).

Flow Cytometry. SNU638 cells (5 x 10^6) were plated in a 100-mm tissue culture dish containing 10 ml of RPMI 1640. After treatment with 10 ng/ml paclitaxel, the cells were harvested and stained with propidium iodide, and then the cell cycle distribution was analyzed by flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, CA) according to Becton Dickinson protocols.

RESULTS
Paclitaxel Cytotoxicity and Apoptosis. All gastric cancer cells used in this experiment were sensitive to paclitaxel (data not shown). This result suggests that paclitaxel is also cytotoxic to gastric cancer cells and can be used as an effective drug against stomach cancer. We also observed paclitaxel-induced apoptosis by DNA fragmentation analysis in gastric cancer cells. The fragmented DNAs were observed on agarose gel
Fig. 3  A, cell cycle analysis of a stable transfectant (SNU638/5) and the control cells treated with paclitaxel. SNU638/neo and a stable transfectant treated with 10 ng/ml paclitaxel were harvested at 0, 24, and 48 h, respectively, and fractions of cell population at G1 phase and G2-M phase were analyzed by flow cytometry. The left column shows SNU638/neo cells containing the neomycin-resistant gene as a control (B, Lane 2). The right column shows SNU638/5 cells overexpressing CDK4 (B, Lane 7). B, CDK4 expression in CDK4-stable transfectants. Protein lysates were resolved by SDS-PAGE, and CDK4 expression was analyzed by Western blotting. Lane 1, SNU638; Lane 2, SNU638/neo; Lanes 3–7, CDK4 stable transfectants.

CDK4 Down-Regulation. To examine the expression of the genes regulated by paclitaxel in paclitaxel-induced apoptosis, we observed the expression of p53, p21, bcl-2, Bax, cyclins, and CDKs in SNU16 and SNU638 cells. The expressions of p53, p21, and Bax were not affected by paclitaxel treatment in SNU16 cells, and the expression of p21, cyclin D1, cyclin E, CDK2, CDK5, and Bax did not change after exposure to paclitaxel in SNU638 cells (data not shown). Interestingly, a decreased expression of CDK4 was observed in gastric cancer cells SNU5, SNU16, and SNU638 and AGS cells that were electrophoresis after 1 day of continuous exposure to paclitaxel at a dosage of 10 ng/ml to 1 μg/ml (data not shown). This apoptosis—induced by paclitaxel—was also detected by flow cytometric analysis. Increasing DNA of less than G1 content appeared 24 h after 50 ng/ml paclitaxel treatment (data not shown).
treated with paclitaxel, although CDK4 expression was not affected by paclitaxel treatment in SNU1 cells, which have shown the highest expression level among all of the tested gastric cancer cell lines (Fig. 1). This result suggests that paclitaxel induces p53-independent apoptosis and the CDK4 down-regulation may be involved in paclitaxel-induced cytotoxicity in gastric cancer cells.

G1 Arrest by Paclitaxel. Paclitaxel treatment has been known to cause an arrest at G2-M phase of the cell cycle (15–18). In this study, we showed that paclitaxel induced a decreased expression of CDK4, which is necessary for the transition from G1 phase to S phase during the cell cycle.

To examine the effect of paclitaxel-induced CDK4 down-regulation on the cell cycle progression of gastric cancer cells, the cell cycle distributions of paclitaxel-treated and untreated controls were determined by flow cytometric analysis. Asynchronously proliferating cells were treated with 10 ng/ml paclitaxel. As shown in Fig. 2, paclitaxel-treated SNU1, SNU5, and SNU16 cells began to show G2 arrest. However, SNU5 and SNU16 cells, in which CDK4 down-regulation was induced by paclitaxel treatment, also displayed an arrest at G1-S phase. This cell cycle arrest at G1-S phase is thought to be induced by CDK4 down-regulation.

To investigate the correlation between CDK4 down-regulation by paclitaxel and cell cycle arrest at G1-S phase, we prepared CDK4-overexpressing cells (Fig. 3B). Enhanced expression of CDK4 in the cells can abrogate the paclitaxel-induced CDK4 down-regulation, which results in preventing the cell cycle arrest at G1-S phase. Proliferating SNU638/neo cells (Fig. 3B, Lane 2) and SNU638/5 cells (Fig. 3B, Lane 7) were treated with 10 ng/ml paclitaxel. This paclitaxel treatment resulted in an arrest at G2-M phase in both cell lines (Fig. 3A). However, paclitaxel-treated SNU638/neo cells showed a concomitant arrest at G1-S phase also. Fractions of cell population (in %) in G1 versus G2-M were changed from 64:18 to 27:58 after 24 h treatment with paclitaxel. In contrast, SNU638/5 cells, which showed increased expression of CDK4, did not block at G1-S phase after paclitaxel treatment. After 48 h, the proportion of cells at G1 phase and G2-M phase (the percentage in G1: G2-M, 31:49) did not significantly change in SNU638/neo cells. For SNU638/5, the proportion of DNA (thought to be apoptotic DNA fragments) of less than G1 content appeared after 48 h of paclitaxel treatment. This result demonstrates that the enforced expression of CDK4 abrogates the paclitaxel-induced G1 arrest.

To measure more precisely the magnitude of the paclitaxel-induced G1 and G2 arrests in CDK4-overexpressing cell lines, an increase in the G2-M fraction after treatment with 5 ng/ml paclitaxel was analyzed in SNU638, SNU638/1, and SNU638/5 cells. SNU638/1 cells showed higher expression of CDK4 compared with the parental cells. However, the level of CDK4 expression was lower than that of SNU638/5 cells, and 12 h after paclitaxel treatment, the SNU638/1 cells began to progress into S and G2-M phases (Fig. 4). SNU638/1 and SNU638/5 cells showed further progression into S and G2-M phase compared with the parental cells (Table 1). The proportion of G1-S phase in SNU638/1 cells was lower than that in SNU638 cells but was higher than that in SNU638/5 cells after paclitaxel treatment.

To confirm the above observations, an additional cell cycle progression assay was done by the measurement of [3H]thymi-
dine incorporation. After serum starvation-arrested cells were stimulated by the addition of media containing serum without and with paclitaxel, the cell cycle progression was monitored. As shown in Fig. 5, paclitaxel treatment induced inhibition of DNA synthesis in SNU638 cells. However, there was no remarkable inhibition of DNA synthesis in SNU1 cells, in which arrest at G1-S phase was not induced by paclitaxel treatment. These data suggest that paclitaxel-induced G1 arrest is associated with decreased expression of CDK4.

**DISCUSSION**

Paclitaxel has been known to be an effective anticancer agent for various cancers. It prevents the completion of mitosis, resulting in a cell cycle arrest at G2-M phase and apoptosis (15–19). Several genes including bcl-2, c-raf-1, and p21 have been studied to define their involvement in paclitaxel-induced apoptosis (21–24). Among these genes, bcl-2 phosphorylation and its inactivation is likely to be the major factor in the process of paclitaxel-induced apoptosis. We tested cell cytotoxicity that was caused by paclitaxel in six Korean gastric cancer cell lines. All of the cell lines were sensitive to paclitaxel treatment, although SNU1 cells were a slightly insensitive to paclitaxel treatment compared with the other cells (data not shown). This result demonstrates that paclitaxel is very effective in killing gastric cancer cells and can be used as an anticancer drug for gastric cancer. That has also been proven by recent reports from several clinical studies. We have tried the clinical application of paclitaxel as a treatment for Korean gastric cancer patients. Our results showed that combination therapy with paclitaxel, 5-fluorouracil, and cisplatin was effective with a response rate of 50% (25).

The aberrations of the CDK4 gene have suggested an involvement in oncogenesis. Amplification and increased expression of CDK4 were observed in glioblastomas and sarcomas (26), and the mutation that disrupts p16 binding was detected in melanoma (27). Deregressions of this pathway derived from these alterations had suggested a common step in the multistep progression of sporadic malignant melanomas (28, 29). We also screened CDK4 overexpression in surgically resected gastric tissues by Western blot analysis. Three of 11 stomach cancer tissues showed CDK4 overexpression compared with the normal matched mucosa. CDK4 overexpression was also detected in 5 of 7 gastric cancer cell lines (data not shown). This CDK4 overexpression can cause an uncontrolled growth of stomach cells, resulting in tumor progression. CDK4 alterations may be one of the factors affecting oncogenesis in stomach cells.

In this study, we showed that CDK4 down-regulation by paclitaxel treatment was correlated with cell cycle arrest at G1-S phase in gastric cancer cells. CDK4-overexpressing SNU638 cells were less arrested at G1-S phase by paclitaxel treatment compared with the parental SNU638 cells. Moreover, the abrogation of paclitaxel-induced CDK4 down-regulation by enforced expression of CDK4 induced the cell cycle progression from G1 to S and G2-M phase, demonstrating that paclitaxel-induced CDK4 down-regulation causes cell cycle arrest at G1-S phase. As shown in Fig. 3A, increased cell proportion at G1-M phase and susceptibility to apoptosis by paclitaxel treatment were observed in CDK4-overexpressing SNU638 cells. Increased cell susceptibility to apoptosis by paclitaxel treatment in SNU638/5 cells seems to be caused by enforced expression of CDK4. Enforced expression of CDK4 conferred a release from G1 arrest on cell cycle and permitted an S-phase entry of cell cycle. This entry to S phase seems to render the cells more sensitive to apoptosis. From our data, we suggest that com-

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<td>Percent of cells in phase of cell cycle</td>
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<tr>
<td></td>
<td>G0-G1</td>
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<tr>
<td>Cells</td>
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</tr>
<tr>
<td>SNU638</td>
<td>53.2</td>
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Fig. 5 The inhibition of cell cycle progression by paclitaxel in SNU638 (A) and SNU1 (B) cells. The cells were plated at the density of 2 x 10^4 cells/well. The cells were cultured in the absence of serum for 3 days. RPMI 1640 was re-added in the presence (○ and △) and the absence (●) of paclitaxel (5 ng/ml). The cell cycle progression was monitored by the measurement of [3H]thymidine incorporation.
pounds that disrupt paclitaxel-induced G₁ arrest may make tumor cells more sensitive to paclitaxel. The importance of CDK4 down-regulation in paclitaxel-induced apoptosis remains to be elucidated.

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