The growth inhibition of liver cancer cells by paclitaxel and the involvement of extracellular signal-regulated kinase and apoptosis

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Abstract. Paclitaxel is a chemotherapeutic drug applied for the treatment of breast and non-small cell lung cancers. However, the biological effects of paclitaxel on hepatocellular carcinoma (HCC) are undefined. We examined these points by using the human HCC cell lines, and found that paclitaxel inhibited the growth of HCC cells and blocked the cell cycle at the G2/M phase. The cell death was partially mediated by apoptosis, because caspases were weakly activated and the cell death was partially rescued by a pan-caspase inhibitor. Paclitaxel activated extracellular signal-regulated kinase (ERK), and when ERK was inhibited by a mitogen-activated ERK-regulating kinase inhibitor, the cell death and cell cycle arrest induced by paclitaxel were rescued, demonstrating that paclitaxel inhibited the cellular growth via the ERK signaling pathway. Our data are promising for the application of paclitaxel in the treatment of patients with HCC.

Introduction

Paclitaxel, originally isolated from the bark of the yew tree, is a chemotherapeutic drug utilized for the treatment of patients with a variety of tumors, including breast, gastric, ovarian, and non-small cell lung carcinomas (1). Paclitaxel has been demonstrated to exert an anti-proliferative effect by binding and stabilizing microtubles, leading to cell cycle arrest at the G2/M phase, and inducing apoptosis in a variety of cancer cells derived from ovary (2), head and neck (3), and esophagus (4). The mechanisms of paclitaxel-induced apoptosis and the roles of mitogen activated protein kinase (MAPK) are different depending on the cellular context, including activation of extracellular signal-regulated kinase (ERK) (5), c-jun N-terminal kinases (JNK) (6), and p38MAPK (7).

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death in the world. Although intrahepatic chemotherapy and systemic drugs including gemcitabine and octreotide have been tested for patients with advanced HCC (8), the prognosis of these patients is not favorable yet. Therefore, the development of new drugs for the treatment of advanced HCC is necessary.

Although paclitaxel has been challenged *in vivo* in a rabbit liver tumor model (9) and patients with HCC (10), information of the cytotoxic effect of paclitaxel in HCC cells is limited (11-13). We therefore sought to clarify the biological effects of paclitaxel and those mechanisms in HCC cells.

Materials and methods

Materials. Antibodies against the phosphorylated form of ERK at the Thr202/Tyr204 residues and ß-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A phosphorylation-independent antibody for ERK and anti-caspase-3 and -9 antibodies were purchased from Cell Signaling technology, Inc. (Beverly, MA, USA). An anti-poly(ADP-ribose) polymerase (PARP) antibody was purchased from BD Pharmingen (San Diego, CA, USA). Secondary anti-mouse and anti-rabbit horseradish peroxidase antibodies were obtained from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA).

Paclitaxel was purchased from Sigma-Aldrich. U0126 [a mitogen-activated ERK-regulating kinase (MEK) inhibitor] was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Z-VAD-FMK (a pan-caspase inhibitor) was purchased from Kamiya Bio-medical Company (Seattle, WA, USA). These compounds were dissolved in dimethyl sulfoxide (DMSO) with the final concentration never exceeding 0.1%.

Cell lines and tissue culture. Human HCC cells (Hep3B, HepG2, HLF, and Huh6) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Cosmo Bio Co., Ltd., Tokyo, Japan) at 37˚C in a 5% CO_2 incubator.

Cell viability. Cell viability was assessed by modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

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Abbreviations: ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; MEK, mitogen-activated ERK-regulating kinase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase

Key words: paclitaxel, extracellular signal-regulated kinase, apoptosis, liver cancer

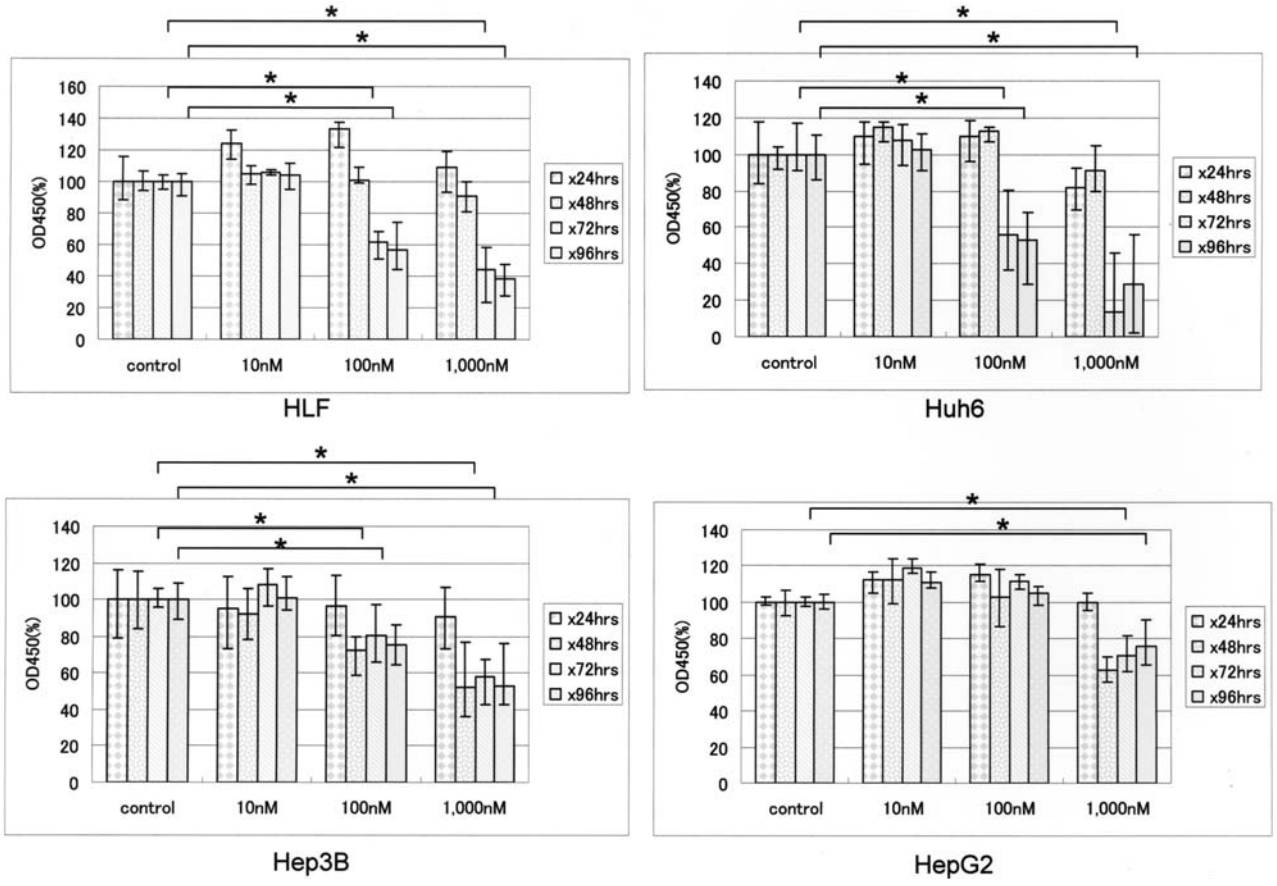

Figure 1. Effects of paclitaxel on the proliferation of liver cancer cells. After HCC cells (Hep3B, HepG2, HLF, and Huh6) were treated with escalating doses (10, 100, and 1000 nM) of paclitaxel for 24, 48, 72, and 96 h, MTT assays were performed. These experiments were repeated at least three times and data are expressed as the mean ± SE. *P<0.01 compared with the controls.

assays (Cell Counting Kit-8, Dojindo Corp., Kumamoto, Japan) following the supplier's protocol. Briefly, cells were grown in 96-well plates at 37˚C in a 5% CO_2 incubator and treated with compounds. After 10 μl of reagent was added, OD_{450} was measured using a microplate reader (Dade Behring Inc., Deerfield, IL, USA). The experiments were repeated at least three times, and the data are expressed as the mean ± SE.

Hoechst 33258 staining. Cells grown in 8-well chamber slides (Nalge Nunc Corp., IL, USA) were fixed in methanol/acetic acid (3:1) solution for 15 min, washed three times with phosphate-buffered saline (PBS) for 5 min, and stained with Hoechst 33258 solution (Sigma-Aldrich) for 20 min light-protected. The stained cells were observed under a fluorescence microscope.

Flow cytometry. Cell pellets were washed with PBS containing 1% FBS, fixed in 70% ethanol, stained with 0.5 mg/ml propidium iodide (Sigma-Aldrich) containing 3 Kunitz RNase (NIPPON Gene Co., Ltd., Tokyo, Japan), and then analysis was performed with a flow cytometer (EPICS-XL, Beckman Coulter, Inc. Miami, FL, USA).

Total protein preparation and Western blotting. Total protein preparation from cells and Western blotting were performed as described previously (14). Briefly, cells were lysed in RIPA buffer [0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM ethylenediaminetetraacetate (EDTA), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor mixture tablet (Roche Diagnostics, Mannheim, Germany)] for 10 min on ice. The total cell lysate (10 μg) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp., Bedford, MA, USA). After blocking with 5% non-fat milk in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween-20), the membrane was probed with a primary antibody overnight at 4˚C, incubated with an anti-mouse or anti-rabbit horseradish peroxidase antibody for 1 h at room temperature, and then the signal was visualized by ECL solution (Amersham Pharmacia Biotechnology).

Statistical analysis. Data are presented as the mean values (columns) ± SE (bars). The data of MTT assays were analyzed using the paired t-test to assess differences between experimental groups. Statistical significance was inferred at P<0.05.

Results

Paclitaxel shows anti-proliferative effect against human liver cancer cells. We first evaluated the effect of paclitaxel on the growth of HCC cells. Because clinically achievable concentrations of paclitaxel are <10 μM (15), we administered paclitaxel at doses of 10, 100, and 1000 nM. Although 10 nM

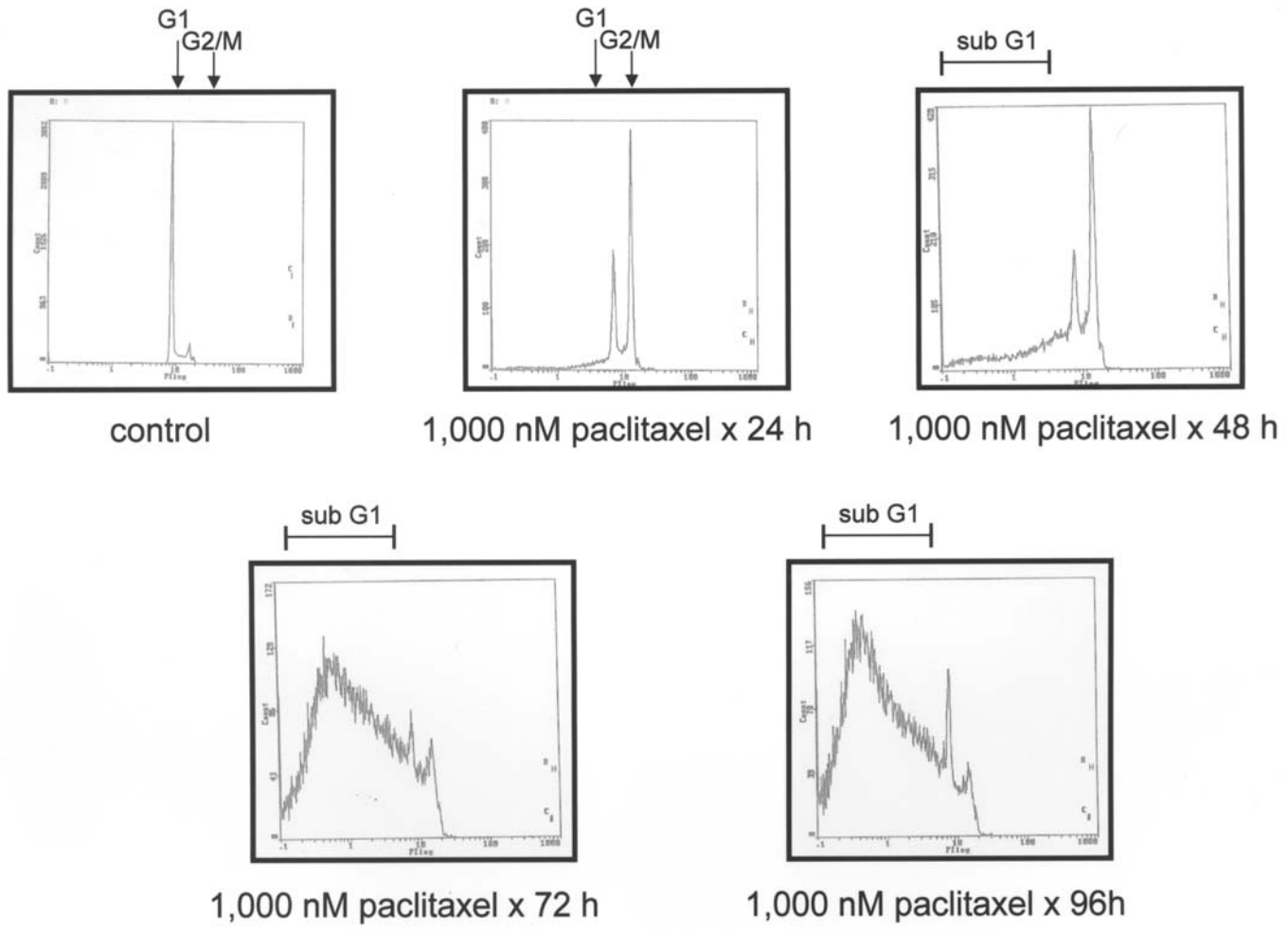

Figure 2. Flow cytometric analysis of HLF cells after the paclitaxel treatment. HLF cells were treated with 1000 nM paclitaxel for 24, 48, 72, and 96 h and flow cytometric analysis was performed. Similar results were obtained in Hep3B, HepG2, and Huh6 cells (data not shown).

paclitaxel did not significantly inhibit the proliferation of HCC cells at any time points, 100 nM paclitaxel significantly inhibited the growth of Hep3B, HLF, and Huh6 cells after 72 and 96 h (Fig. 1) and 1000 nM paclitaxel inhibited the growth of all HCC cell lines. These results demonstrate that paclitaxel inhibited the growth of HCC cells at clinically relevant doses.

Induction of apoptosis and blockade of the cell cycle by paclitaxel in liver cancer cells. We next performed flow cytometric analysis in HCC cells. Paclitaxel blocked the cell cycle at the G2/M phase in HLF cells (Fig. 2) as well as in Hep3B, HepG2, and Huh6 cells (data not shown), in agreement with previous reports (16). In addition, the subG1 fraction appeared after the treatment with paclitaxel (Fig. 2), implying the induction of apoptosis.

Nuclear morphology after the paclitaxel treatment. For confirmation of the induction of apoptosis by paclitaxel, nuclear morphology was examined by nuclear staining with Hoechst 33258. When HLF cells were treated with 100 nM of paclitaxel for 96 h, nuclear shrinkage, condensation, and fragmentation, indicative of apoptosis, were observed (Fig. 3).

Paclitaxel activates ERK in liver cancer cells. Since the ERK signaling pathway has been involved in the regulation of cellular growth and apoptosis (17), we examined the modulation of ERK activity when HCC cells were treated with paclitaxel. ERK was significantly activated by the treatment with 1000 nM paclitaxel for 72 h in HLF cells (Fig. 4, left panel). The ERK activation started to appear after 24 h, was sustained until 72 h, and then decayed after 96 h of the treatment (Fig. 4, right panel). Similar results were obtained in Hep3B, HepG2, and Huh6 cells (data not shown). These results demonstrate that paclitaxel induced the prolonged activation of ERK in HCC cells.

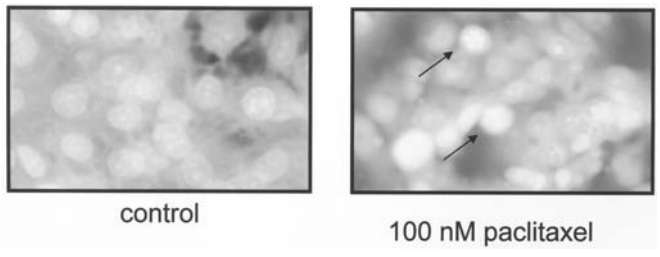

Figure 3. Nuclear morphology of HLF cells after the paclitaxel treatment. HLF cells were treated with 100 nM paclitaxel for 96 h and nuclear morphology was examined by Hoechst 33258 staining under a fluorescence microscope (x400 magnification). Similar results were obtained in Hep3B, HepG2, and Huh6 cells (data not shown).

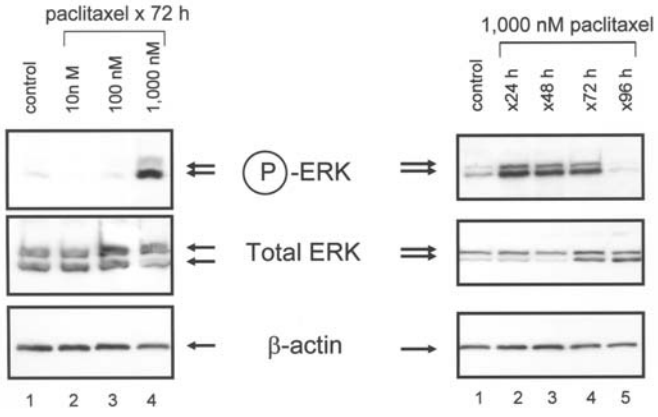

Figure 4. Effects of paclitaxel on ERK activity. After HLF cells were treated with paclitaxel, the dose effect on the phosphorylation of ERK (left, upper panel) and time course of the phosphorylated ERK (right, upper panel) were examined by Western blot analysis. Total expression levels of ERK were not significantly modified by the paclitaxel treatment (middle panel). Similar results were obtained in Hep3B, HepG2, and Huh6 cells (data not shown).

Activation of caspase-3 and -9 by paclitaxel. We next investigated the involvement of caspases in the paclitaxel-induced apoptosis in HCC cells. The cleaved product of PARP, a hallmark of apoptosis, was observed after HLF cells were treated with 1000 nM paclitaxel for 72 h (Fig. 5). Because caspase-3 and -9 are representative caspases responsible for the PARP cleavage (18), we evaluated the activation of these caspases by Western blot analysis. The full-length caspase-3 and -9 were decreased, and cleaved form products of these caspases appeared after HLF cells were treated with 1000 nM paclitaxel for 72 h (Fig. 5, left panel).

We evaluated whether the growth inhibition by paclitaxel could be reversed by pre-treatment with a pan-caspase inhibitor, Z-VAD-FMK. The cell-death induced by paclitaxel was partially rescued by Z-VAD-FMK (Fig. 5, right panel). These results demonstrate that activation of caspase-3 and -9 are in part involved in the paclitaxel-induced apoptosis in HCC cells.

Roles of activated ERK by paclitaxel. We further investigated the roles of activated ERK by paclitaxel treatment in HCC cells. When HLF cells were pre-treated with 10 μM U0126 prior to the paclitaxel treatment, which inhibited the ERK phosphorylation induced by paclitaxel (Fig. 6, upper, left panel), the G2/M arrest and the subG1 fraction induced by paclitaxel were mostly abrogated (Fig. 6, lower panel), demonstrating the roles of ERK on the G2/M arrest and apoptosis induced by paclitaxel. However, the cellular growth was rescued in part by U0126 (Fig. 6, upper, right panel).

Discussion

We showed that paclitaxel inhibited the growth of HCC cells at doses of 100 and 1000 nM. Since a peak plasma concentration

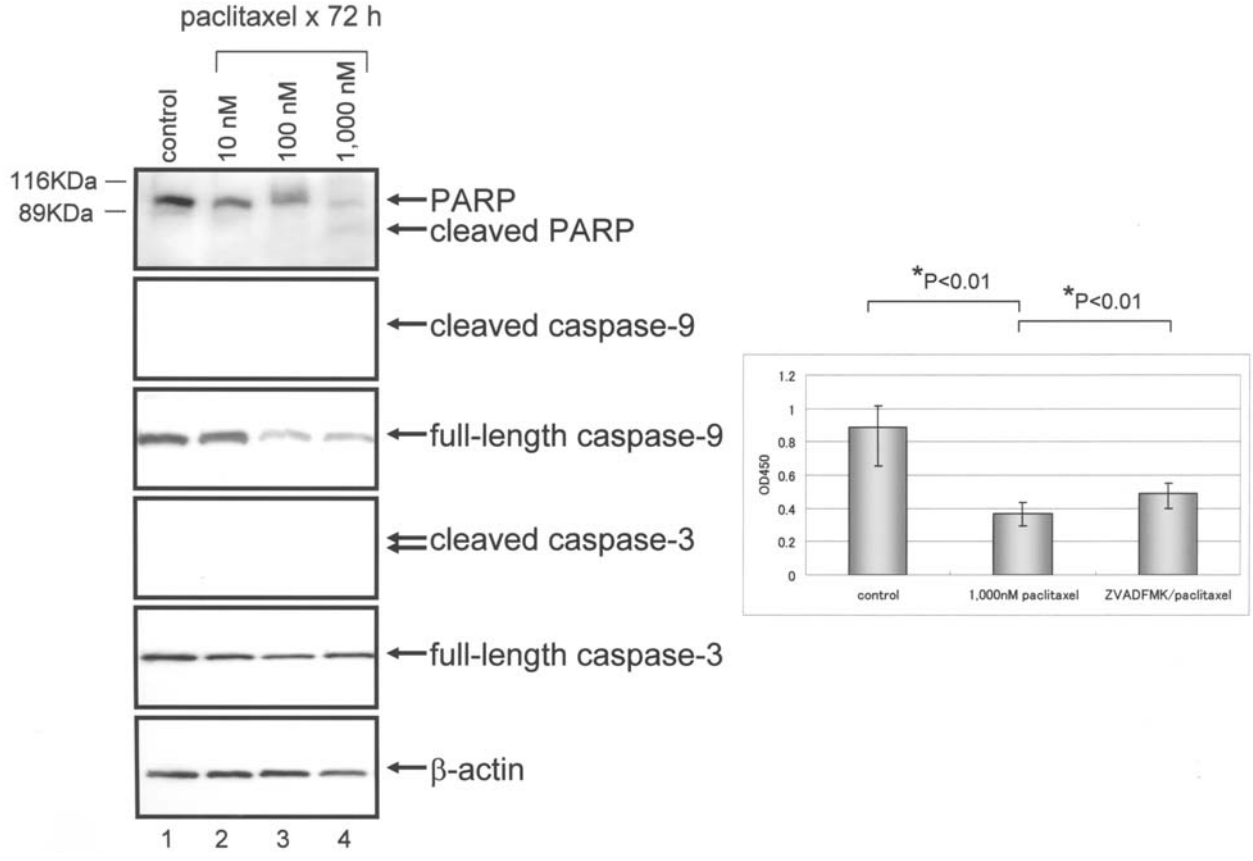

Figure 5. Activation of caspase-3 and -9 in the cell death by paclitaxel. Western blot analysis with antibodies against PARP, caspase-3, and -9 was performed using total cell lysates of HLF cells treated with escalating doses of paclitaxel for 72 h (left panel). MTT assays were conducted after 25 μM pan-caspase inhibitor, Z-VAD-FMK, was added to HLF cells 30 min prior to the paclitaxel treatment (right panel). MTT assays were repeated three times and data are expressed as the mean ± SE. *P<0.01 compared with the controls.

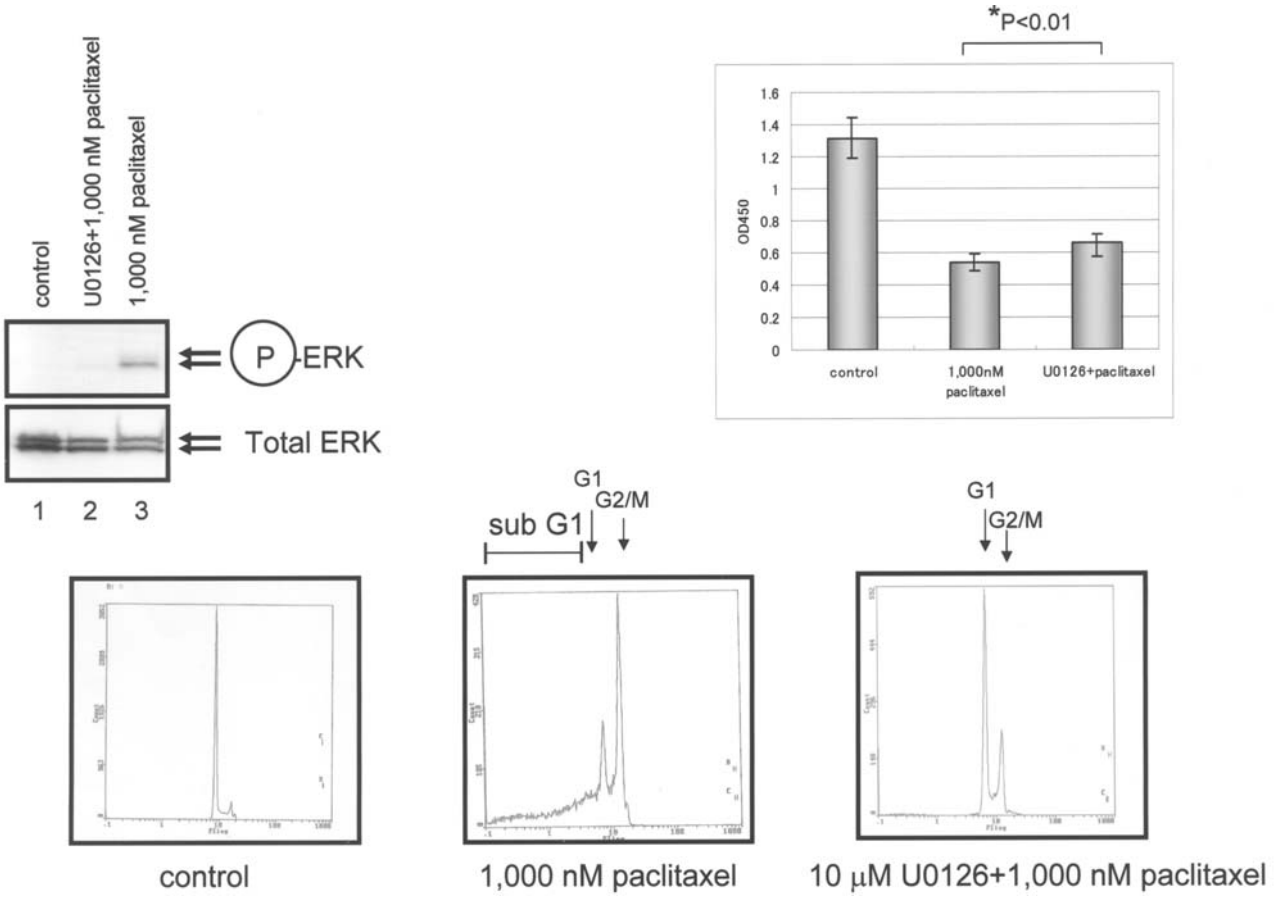

Figure 6. Roles of ERK on HLF cells treated with paclitaxel. Abrogation of the phosphorylated ERK was confirmed by pre-treating HLF cells with 10 μM U0126 prior to the treatment with 1,000 nM paclitaxel (upper, left panel). Effects of pre-treatment with 10 μM U0126 on the cellular growth (upper right panel), and the cell cycle and apoptosis (lower panel) were evaluated by MTT assays and flow cytometric analysis, respectively.

of paclitaxel in cancer patients is approximately 10 μM (15), our data are clinically relevant.

Much effort has been made to improve the prognosis of patients with advanced HCC, including continuous intrahepatic administration of 5-fluorouracil and cisplatin (19) and a combinatory treatment of these drugs with interferon-α (20). Although paclitaxel has been widely used for patients with breast, gastric, ovarian, and non-small-cell lung carcinomas (1), the efficacy of paclitaxel in the treatment of patients with HCC has not yet been established. In this report, we evaluated the biological effects of paclitaxel on HCC cells and explored their mechanisms.

Our data obtained from nuclear staining and flow cytometric analysis implied that the death of HCC cells induced by paclitaxel was mediated by apoptosis. However, a pan-caspase inhibitor, Z-VAD-FMK, rescued the cell death of paclitaxel in part, and the activation of caspase-3 and -9 by paclitaxel was weak when evaluated by Western blot analysis. Because there are some previous reports demonstrating caspase-independent mechanisms in paclitaxel-induced apoptosis (4,13,21), we speculate that a caspase-independent mechanism, a so-called slow-cell death, which involves non-caspase proteases such as calpains and cathepsins (22,23), may be involved in the cell death induced by paclitaxel in HCC cells.

ERK is one of the MAPK signaling molecules involved in the regulation of cell growth, cell cycle (24), and sensitivity to chemotherapeutic agents (25). ERK activation exerts either an anti-apoptotic or a pro-apoptotic influence depending on the cellular context (26). In our experiments, the function of activated ERK appeared to be pro-apoptotic, because pre-treatment with an MEK inhibitor, U0126, mostly abrogated the subG1 fraction induced by paclitaxel, and rescued the cell death in part.

Based on our data demonstrating the anti-proliferative effect of paclitaxel on HCC cells, paclitaxel may be useful as a new tool for treating patients with advanced HCC. However, paclitaxel needs to be utilized with caution when applied to patients with complications such as chronic liver diseases, because paclitaxel is metabolized in the liver and those patients are at increased risk of paclitaxel toxicities (10).

Acknowledgements

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