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PD98059 Induces the Apoptosis of Human Cervical Cancer Cells by Regulating the Expression of Bcl2 and ERK2

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PD98059 is the specific inhibitor of extracellular signaling-regulated kinase (ERK) kinase (MEK). ERK is involved in a mitogen-activated protein kinase (MAPK) cascade controlling cell growth and differentiation. Although the inhibition of ERK is known to induce cell death in various cell lines, this effect is still controversial and the role of PD98059 on the death of HeLa S_3 cells, a subclone of the cervical cancer cell line, is not well understood. The apoptosis of HeLa S_3 cells increased after the treatment of 50 μM PD98059. The induction of apoptosis by PD98059 was occurred in a time- and a dose-dependent manners. The expression of Bcl-2 was reduced in accordance with decrease of ERK2 expression. Taken together, these results indicate that PD98059 has a cytotoxicity in HeLa S_3 cells and it may be used as a potential target for the treatment of cervical cancer.

Key Words: PD98059, Apoptosis, Bcl-2, HeLa S_3 cell

INTRODUCTION

Several signal transduction pathways are activated by the stimulation of cells with growth factors and cytokines, or exposure to cellular stresses and have specific physiological roles (Alessi et al., 1995). The mitogen activated protein kinase (MAPK) are involved in various cell stimulations and are activated within the protein kinase cascades called MAPK cascade. MAPK cascade contains p21 and the protein kinases c-Raf, MAP kinase kinase-1 (MAPKK1) and MAPKK2, and p42 and p44 MAP kinases (p42, p44). The sequential activation of these MAPKs phosphorylates a variety of proteins *in vivo* including MAP kinase-activated protein (MAPKAP) kinases, and induces the proliferation or differentiation of several cells (Marshall, 1995).

Specific inhibitors of particular protein kinases are small cell-permeant molecules. These inhibitors may have therapeutic potential as anti-cancer, or anti-inflammatory agents, or as immunosuppressants (Fry et al., 1994). PD98059 (PD) was identified as a noncompetitive inhibitor of MAP kinase kinase (MAPKK) and inhibited the constitutively active mutant (MAPKK1 (S217E, S221E)) in which the serine residues phosphorylated by c-Raf had been mutated to glutamic acid (Dudley et al., 1995).

Cervical cancer has the leading incidence of female cancers. In this cancer, the signaling activities of survival-associated proteins increase (Abraham et al., 2005). Especially, the anti-apoptotic Bcl-2 has been implicated in a number of cancers and involved in resistance to conventional cancer treatment (Yang and Chang, 2011). Although surgery and various chemotherapies for the treatment of cervical cancer have been developed, most response rate is still too low. In addition, these therapies have side effects with toxicity (Yuan et al., 1997). Therefore, there is a pressing need for the development of new agent for the treatment of cervical cancer.

In the present study, we investigated the anti-proliferative effects of PD on the human cervical cancer cell line, HeLa S_3 cells. We examined the cell death of HeLa S_3 cells treated with PD in a time- and a concentration-dependent manners.

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MATERIALS AND METHODS

Reagents

PD (Calbiochem, San Diego, CA) was dissolved in DMSO to prepare the stock solutions (10 mM). Dulbeco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Life Technologies, Inc. (Gaithersburg, MD). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit were purchased from BD biosciences (San Diego, CA). Trypan blue stain solution, anti-Bcl-2 and anti-ERK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

HeLa S_3 cells were a subclone of the cervical cancer cell line and were purchased from American Type Culture Collection (Rockville, MD, USA). These cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), and were incubated at 37℃ in 5% CO_2.

Trypan blue exclusion test

Cell viability was determined by the dye exclusion test. A cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. The cell survival index was calculated as follows: viable cells (%) = total number of viable cells per ml of aliquot/total number of cells per ml of aliquot * 100.

Cell apoptosis

For measurement of the apoptosis, the cells were incubated with the FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic cells were analyzed by flow cytometry using CellQuest software and were defined as the cells in the right quadrant that stained positive for Annexin V with/without PI. To analysis, 10,000 events were collected for each sample.

Western blotting

The cells were washed with ice-cold PBS and lysed with lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). The cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. The blots in membrane were incubated with anti-Bcl-2 antibody and thereafter developed using an enhanced chemiluminescence detection system. The same blot was stripped and reprobed with anti-ERK2 antibodies for use as internal control. Quantification of Western blots was performed using Quantity One software (Bio-Rad Laboratory, Inc.).

RESULTS

PD98059 induces apoptosis of HeLa S_3 cells in a time-dependent manner

To determine the effect of PD on the viability and the death of HeLa S_3 cells, we examined the viability and apoptosis/necrosis of the cell induced by PD. The cells were treated with 50 μM PD and then incubated for 6, 12, 18, 24 or 48 hours at 37℃. Cell viability was analyzed by the trypan blue exclusion test. Apoptosis and necrosis of cells were assessed by FITC-conjugated annexin V and PI staining using flow cytometry. Annexin V is a marker for phosphatidylserine (PS), which exposed on plasma membrane at the initial stage of apoptosis. PI is a marker for membrane-permeability in the late-apoptosis and necrotic cells. Apoptotic cells were defined as all annexin V- positive cells. The percentages of apoptotic cells in HeLa S_3 cells treated with 50 μM PD increased until 48 hours incubation (Fig. 1). On the other hand, the percentages of viable cells decreased from 92.8% to 57.8% (Fig. 1). These results indicate that PD induces the cell death of HeLa S_3 cells via apoptotic pathway.

PD98059 alters the expressions of Bcl-2 protein in HeLa S_3 cells

To examine the alteration of apoptosis-associated protein,

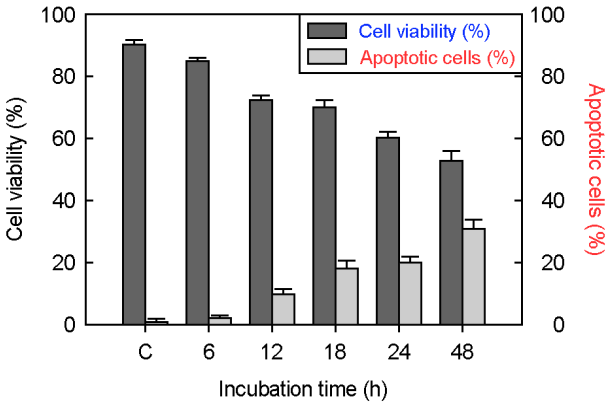

Fig. 1. Effect of 50 μM PD98059 on apoptotitc cells, and cell viability in HeLa S_3 cells. HeLa S_3 cells were incubated with 50 μM of PD98059 (PD) for the indicated times. C represents the control. After treatment with PD, viable cells were determined by trypan blue exclusion test as described in the materials and methods section. The apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry as described in the materials and methods section. The percentage of apoptotic cell in total cell population was included all annexin V binding cells. Data are expressed as the means ± SD in three individual experiments.

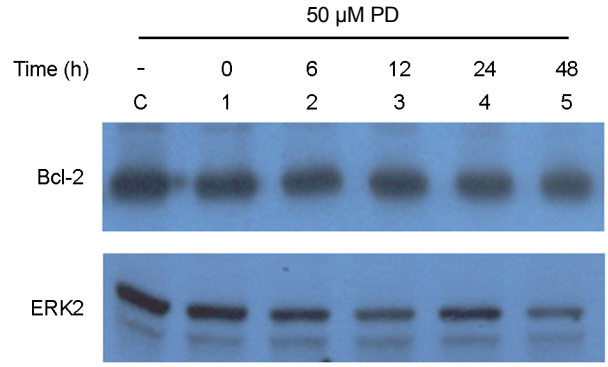

Fig. 2. Western blot analysis of Bcl-2 and ERK2 expresion in HeLa S_3 cells treated with 50 μM PD98059. HeLa S_3 cells were treated with 50 μM PD98059 (PD) and then incubated for the indicated times (lanes 1~5). Lane C represents the control. Harvested cells were lysed and were performed Western blotting as described in the materials and methods section. The expression of Bcl-2 and ERK2 was detected with anti-Bcl-2 and anti-ERK2 antibodies.

Western blot was performed to estimate the levels of Bcl-2 and ERK2 protein. When HeLa S_3 cells were treated with 50 μM PD, PD slightly suppressed the level of Bcl-2, a anti-apoptotic signal protein (Fig. 2). In addition, PD decreased the level of ERK2 protein (Fig. 2).

PD98059 induces apoptosis of HeLa S_3 cells in a concentration-dependent manner

Fig. 3 shows the percentages of apoptotic cells and cell viability in HeLa S_3 cells treated with 100, 200 and 500 μM PD and then incubated for 6, 24 or 48 hours at 37℃. As shown in the results, there is remarkable difference among the cells treated with various concentration of PD. 500 μM of PD considerably led apoptosis of these cells at 6, 24 and 48 hours. These results indicate that PD98059 induces the apoptosis of HeLa S_3 cells in a time- and a dose-dependent manners (Fig. 3).

PD98059 decreases the Bcl-2 protein level of HeLa S_3 cells in a concentration-dependent manner

We examined the apoptotic effect of PD on HeLa S_3 cells in a concentration-dependent manner. The cells were treated with 100, 200 or 500 μM of PD for 6, 24 or 48

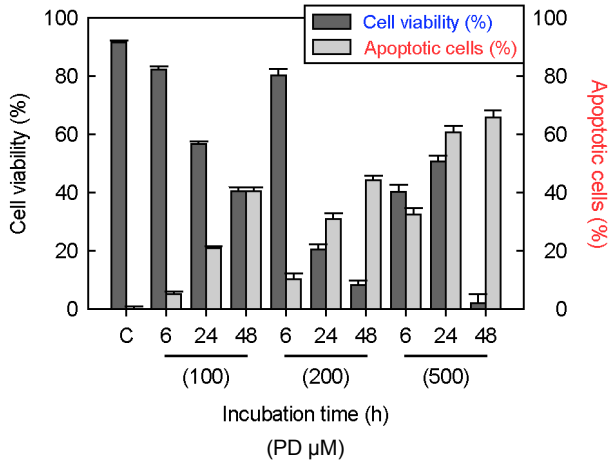

Fig. 3. Effect of various concentration of PD98059 on apoptotic cells and cell viability in HeLa S_3 cells. HeLa S_3 cells were incubated with 100, 200 or 500 μM of PD98059 (PD) for the indicated times. C represents the control. After treatment with PD, viable cells were determined by trypan blue exclusion test as described in the materials and methods section. The apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI using flow cytometry as described in the materials and methods section. The percentage of apoptotic cell in total cell population was included all annexin V binding cells. Data are expressed as the means ± SD in three individual experiments.

hours. When HeLa S_3 cells were treated with PD, the level of Bcl-2 protein continuously decreased, and the level of ERK2 protein also markedly suppressed in a time- and a dose-dependent manners (Fig. 4).

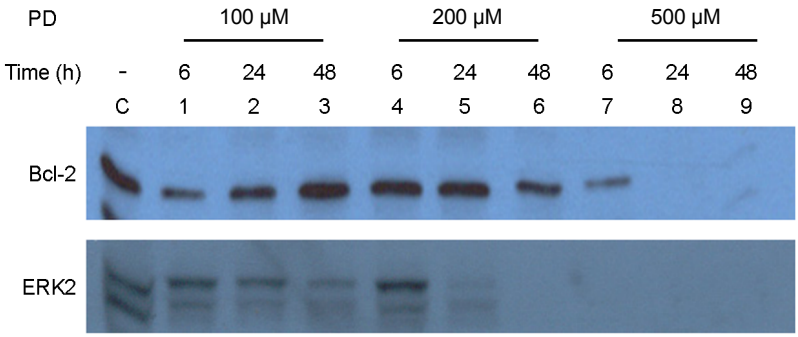

Fig. 4. Western blot analysis of Bcl-2 and ERK2 expression in HeLa S_3 cells treated with various concentration of PD98059. HeLa S_3 cells were treated with 100, 200 or 500 μM PD98059 (PD) and then incubated for the indicated times (lanes 1~9). Lane C represents the control. Harvested cells were lysed and were performed Western blotting as described in the materials and methods section. The expression of Bcl-2 and ERK2 was detected with anti-Bcl-2 and anti-ERK2 antibodies.

DISCUSSION

PD has been shown previously to inhibit the dephosphorylated form of mitogen-activated protein kinase kinase-1 (MAPKK1) and a mutant MAPKK1, which has low levels of constitutive activity (Dudley et al., 1995). Especially, ERK-2 has pro-apoptotic or anti-apoptotic effects in human cervical cancer cells (Pucci et al., 2001; Ying et al., 2011). In the present study, we focused on the cytotoxic activity of PD on the human cervical cancer cell line, HeLa S_3 cells. We demonstrated that PD considerably increased the apoptosis of HeLa S_3 cells via the alteration of intracellular signaling proteins. The MAPK/ERK pathway starts when a growth factor binds to the receptor on the cell surface and includes many proteins, which communicate by adding phosphate groups to a neighboring protein (Orton et al., 2005). A defect in the MAPK/EKR pathway leads to uncontrolled growth in various cancers (Hoshino et al., 1999; Hilger et al., 2002; McCubrey et al., 2007; Sebolt-Leopold, 2008; Wong, 2009). Uncontrolled growth is a necessary step for the development of all cancers (Downward, 2003). In human cervical cancer cells, MAPK/ERK pathway, including AKT (protein kinase B, PKB), ERK-1/2, p38 MAPK and c-Jun N terminal kinase (JNK) are phosphorylated by various carcinogens (Kim et al., 2009). The inhibition of the phosphorylated proteins induces the suppression of tumor cell growth from various cancers. HeLa S_3 cells were increased cell death by 50 μM of PD (Fig. 1), and the cell death induced by PD was occurred in a concentration- and a time-dependent manners (Fig. 1 and Fig. 3). PD is used as a specific inhibitor of the activation MAPK/ERK. PD inhibits the activation of p42 and isoforms of MAP kinase-activated protein kinase-1 in many cells. PD decreased the expression of ERK2 protein in HeLa S_3 cells (Fig. 2 and Fig. 4). These results indicate that PD plays a key role for this pathway in the proliferation of HeLa S_3 cells. In other previously studies, PD also inhibited the cell growth by the suppression of Ras overexpression in KRNK and K-balb cells (Dudley et al., 1995) or the nerve growth factor (NGF)-induced differentiation of PC12 cells (Pang et al., 1995).

Cell death occurs by extracellular factors and induces the intrinsic death pathways and continuously mediates decrease of anti-apoptotic signals and increase of pro-apoptotic signals. In apoptosis-associated signal proteins, the anti-apoptotic Bcl-2 has been implicated in a number of cancers and involved in resistance to conventional cancer treatment. Bcl-2 has been reported to exert its inhibitory effects on apoptosis by blocking the release of cytochrome c and the loss of MMP, and this protein has been acting on the inhibition of cell apoptosis in cancer pathogenesis (Gross et al., 1999). PD-induced death of HeLa S_3 cells causes the suppression of Bcl-2 expression in a concentration- and a time-dependent manners (Fig. 2 and Fig. 4). MAPK/ERK signal protein is involved in the modulation of anti-apoptotic Bcl-2 protein and pro-apoptotic Bcl-2 family proteins in various cells (Tomiyama et al., 2010).

In conclusion, PD induces the cell death via the inhibition of MAPK/ERK pathway in the human cervical cancer cell line, HeLa S_3 cells. The inhibition of MAPK/ERK pathway induced suppression of anti-apoptotic Bcl-2 protein. These results suggest that the PD has a potent apoptosis-inducing activity and it may be useful for various cancer therapies.

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