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Relation of Poly(ADP-ribose) Polymerase Cleavage and Apoptosis Induced by Paclitaxel in HeLa S_3 Uterine Cancer Cells

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Although paclitaxel induces apoptosis of cancer cells, its exact mechanism of action is not yet known. The present study has been performed to determine whether influence of paclitaxel in HeLa S_3 uterine cancer cells. Three assays were employed in this study: cell cytotoxicity, morphological assessments of apoptotic cells (DAPI staining assay), and western blot analysis. The results indicated that paclitaxel has cytotoxic effects in HeLa S_3 cells. Especially, the IC_{50} value of paclitaxel was about 1 μM. And morphological changes (fragmentation) of cells were observed by paclitaxel in HeLa S_3 cells. The flow cytometric analysis of paclitaxel-treated cells indicated a block of G2/M phase. The results that paclitaxel regulates the cell cycle, especially Sub-G_1 phase. Paclitaxel induces apoptosis of HeLa S_3 cells via PARP-dependent fashion, and this apoptosis is related to disappearance of Bcl-2 proteins.

Key words – Paclitaxel, PARP, Bcl-2

Introduction

Cervical cancer is the most frequently diagnosed cancer of females in developing countries and the second most frequent cancer affecting women worldwide [24]. Current treatment modalities such as surgical ablation and/or external radiotherapy intervention remain largely palliative for cervical cancer patients because the disease recurs in a refractory form. Long term disease-free treatment consists of cytotoxic chemotherapeutic agents that kill cancer cells mainly by apoptosis. However, commonly used cytotoxic chemotherapy is largely associated with highly nonspecific cytotoxicity, narrow therapeutic indices, and undesirable side effects [3].

Actin microfilaments, intermediate filaments and microtubules are the major constituents of the cytoskeleton in eukaryotic cells. Cytoskeleton plays an important role in the processes of growth and differentiation. Furthermore, changes in the interaction between different cytoskeleton proteins occur during maturation [26]. Paclitaxel, isolated from *Taxus brevifolia*, is the drug of choice with significant anti-tumor activity toward breast, ovarian cancer, among others [21,40,43]. Paclitaxel promotes microtubule assembly, inhibit microtubule depolymerization, and change microtubule dynamics, resulting in disruption of the normal reorganization of the microtubule network required for mitosis and cell proliferation [1,6]. Therefore, cells treated with paclitaxel are unable to proceed normally through the cell cycle and are arrested in the Sub-G_1 and G_2/M phases, finally leading to apoptotic cell death [28,37,39].

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Once triggered apoptosis proceeds with different kinetics depending on cell types and culminates with cell disruption and formation of apoptotic bodies. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and the uptake of these cells by phagocytes. Apoptotic signaling and execution pathways involve the activation of caspase, which in turn cleave key protein substrates. Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like phosphatidylserine (PS) in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost.

Also, one of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The degradation of DNA in the nuclei of apoptotic cells is accom-

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plished in a number of ways following activation of caspases. Western blots were used to measure protein expression level of several genes related to apoptosis. The enzyme poly (ADP-ribose) polymerase, or PARP, was the first protein identified as a substrate for caspases. PARP is involved in repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) and by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP. The oncogenic protein Bcl-2 which is expressed in membranes of different subcellular organelles protects cells from apoptosis induced by endogenic stimuli. Bax is proapoptotic member of the Bcl-2 family of proteins that is implicated in the pathogenesis of cell death in an increasing number of models of apoptosis both in vivo and in vitro. In particular, Bax has emerged as a mediator of the mitochondrial phase of apoptosis, a process that culminartes in the release of cytochrome c from the intermitochondrial space and the activation of effector caspases. Although much has been learned concerning the metabolism of paclitaxel, a clear understanding of the biochemical bases for its apoptosis-inducing activity have not yet emerged. The purpose of this study is to elucidate the fundamental mechanism in relation to apoptosis induced by paclitaxel in HeLa S_3 cells.

Materials and methods

Cell Culture

The HeLa S_3 human cervical carcinoma cell line was maintained in Dulbecco's modified Eagle's medium (D-MEM) with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2.

Reagents

Paclitaxel were purchased from Sigma (St. Louis, MO) and dissolved in PBS, to give a stock solution of 5 mM. 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenylterazolium bromide (MTT) were purchased from sigma.

Cell Viability Assay

Cell viability was measured by hemocytometer using the trypan blue dye exclusion. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 mins, and more than 2×10^2 cells were scored on a hemocytometer. Viable and nonviable cells were counted by inverted microscopy.

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenylterazolium bromide (MTT) Assay

Cytotoxic effects of paclitaxel were determined by MTT Assay. The cytotoxic effect of ETA (Sigma) in cells was estimated by MTT assay. In the MTT assay, cells were placed in a 96-well plate and incubated for 24 hr. Then cells were treated with various concentrations of paclitaxel. And then, the cells were treated with 1 mg/ml of MTT in growth medium. Cells were incubated at 37°C, 5% CO_2 for 4 hr. The medium was aspirated and the formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cell, were dissolved in 200 μl DMSO (dimethyl sulfoxide). Cell viability was evaluated in comparison to the control culture (taken as 100%) by measuring the intensity of the blue color (OD at 540 nm) by a multi-well reader (Quant, Bio-Tek, Highland Park, USA). The assay was performed in triplicate.

4'-6-Diamidino-2-phenylindole (DAPI) Staining Assay

For DAPI Staining Assay, 1×10^6 cells were plated in 2 ml growth medium in the presence or absence of various concentrations (10 nM-100 μM) of paclitaxel in 6-well plates and cultured at 37°C in 5% CO_2 for 24 or 48 hr. Wash the PBS and add the 4% paraformaldehyde 500 μl, and incubated at 4°C for 1 hr. After washing with PBS, DAPI was added (500 μl). Incubated at 4°C for 5 min and observed by fluorescence microscopy. Apoptotic cells were morphological defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation.

Paclitaxel regulated sub-G1 arrest in cells

Cells were harvested in PBS-EDTA, fixed in cold 70% ethanol, and stored at -20°C. Fixed cells were subsequently washed, treated with 100 μg/ml RNase A, and stained with 50 μg/ml propidium iodide. Analysis of DNA content was performed in a Becton Dickinson FACScan with a minimum of 1×10^4 events collected for analysis using Becton Dickinson Cell Quest software. Cells were sorted based on expression of green fluorescent protein and DNA content was analyzed in these cells.

Annexin V Bind Assay to Detect Apoptosis Cells

After treatment with paclitaxel, the cells were used to determine the translocation of phosphatidylserine to the outer surface of the plasma membrane during apoptosis

using the human phospholipid binding protein, Annexin V, conjugated with fluorescein (Molecular Probes, Inc., Eugene, OR) by flow cytometry as described by the manufacturer. Apoptosis and necrosis were analyzed by quadrant statistics on the propidium iodide-negative, fluorescein isothiocyanate-positive cells, and propidium iodide-positive cells, respectively.

Western Blot Analysis

For western blot analysis, 1×10^4 cells were plated in 2 ml growth medium in the presence or absence of various concentrations (10 nM-100 μM) of paclitaxel in 6-well plates and cultured at 37°C in 5% CO_2 for 48 hr. After harvested, cells were washed with PBS and lysed in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 20 mg/mL aprotinin, 50 μg/mL leupeptin, 1 mM benzaidine, 1 mg/mL pepstatin) for 30 min, followed centrifugation (12,000 rpm, 30 min). Protein content was determined using the bradford assay. Equivalent amounts of protein (30 μg) were resolved by 12% SDS-PAGE, transferred to poly-vinylidene difluoride membrane, and probed with the antibodies. Blots probed for PARP were detected with HRP-linked secondary antibodies and enhanced chemiluminescence western blotting reagents (Amersham Pharmacia Biotech), according to manufacturer's protocols.

Results

Effect of paclitaxel in cell viability

To confirm cell viability of cells, abovementioned trypan blue assay is performed in HeLa S_3 cells. Paclitaxel treatment of HeLa S_3 cells decreased cell viability in a dose-dependent and time-dependent decreased manner (Fig. 1). Therefore, paclitaxel had a significant inhibitory effect on the cell viability of HeLa S_3 cells

Cytotoxic effect of paclitaxel in HeLa S_3 cells

To examine paclitaxel-induced growth arrest and apoptosis in HeLa S_3 cells, we assessed the effect of paclitaxel on survival and proliferation of these cells by treating them with various concentrations of paclitaxel for 24hr or 48hr followed by MTT assays. The results presented in Fig. 2 revealed that paclitaxel at 10, 100nM and 10, 100 μM reduced the proliferation and survival of HeLa S_3 cells in a does-dependent fashion.

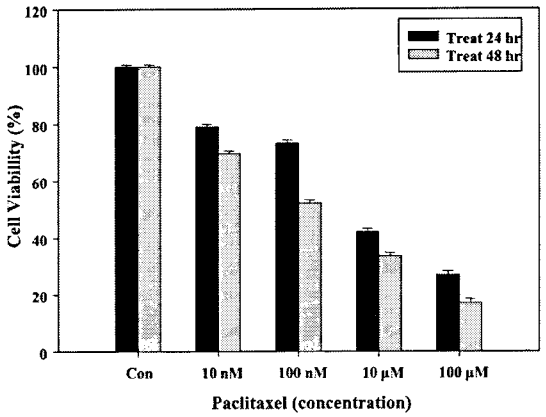

Fig. 1. Effects of paclitaxel in cell viability. HeLa S_3 cells (1×10^4 cells/well in 6-well) were incubated with various concentrations of paclitaxel for 24 hr or 48 hr. Trypan blue-positive cells were considered as nonviable and their percentage was estimated by bright-field microscopy

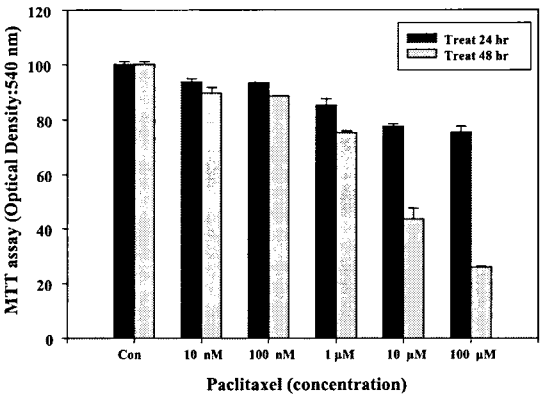

Fig. 2. Cytotoxic effect of paclitaxel in HeLa S_3 cells. HeLa S_3 cells (1×10^4 /well in 48-well plates) were treated with or without increasing concentrations of paclitaxel at 37°C for 24 hr or 48 hr, and cell survival induced by paclitaxel was determined by MTT assay.

Cell viability by paclitaxel in other cancer cells

To confirm cell viability of other cancer cells, abovementioned trypan blue assay is performed in A549 lung cancer cells and B16 melanoma cancer cells. The results presented in Fig. 3 revealed that cell viability of A549 cells or B16 cells reduced in a dose-dependent fashion, respectively.

Morphological change by paclitaxel in HeLa S_3 cells

To confirmed morphological changes (fragmentation) of HeLa S_3 cells by paclitaxel, 4'-6-Diamidino-2-phenylindole (DAPI) staining assay was performed. After treatment with

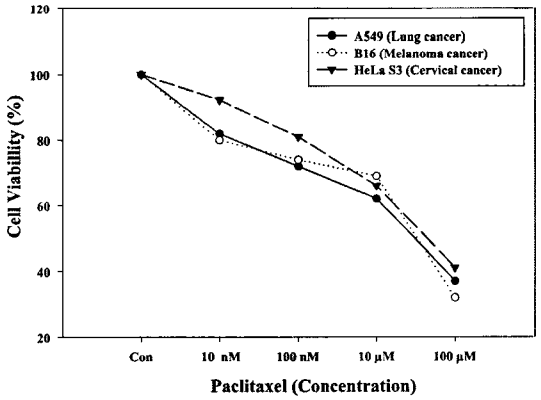

Fig. 3. Effects of paclitaxel in other cancer cell viability. A549 cells and B16 cells were incubated with various concentrations of paclitaxel for 24 hr and 48 hr. Trypan blue-positive cells were considered as nonviable and their percentage was estimated by bright-field microscopy.

paclitaxel for 24 hr, observed morphological change in concentration of 10 and 100 μM. Also after 48 hr, observed morphological change. And intensity of fluorescence increased in a does-dependent manner (Fig. 4).

Paclitaxel regulated Sub-G_1 arrest in HeLa S_3 cells

Paclitaxel, through its stabling effects on microtubules, induces Sub-G_1 arrest. To determine whether Paclitaxel controlled Sub-G_1 arrest in HeLa S_3 Cells, flow cytometric cell cycle analyses were performed following the PI staining of nuclei. Fig. 5 shows the results from a representative experiment in which HeLa S_3 cells were incubated for 24 hr or 48 hr with various concentration of paclitaxel. In this reports confirmed that Sub-G_1 arrest is enhanced in a dose-dependent manner.

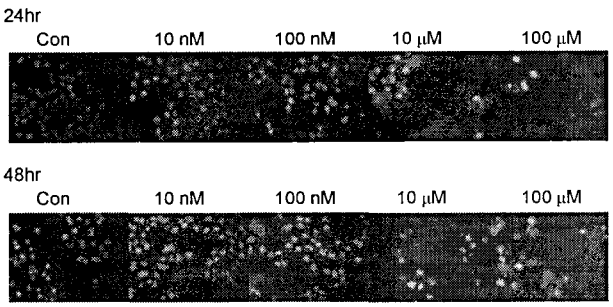

Fig. 4. Morphological change by paclitaxel in HeLa S_3 cells. For DAPI staining assay, 1 × 10^4 cells were plated in 2 ml growth medium in the presence or absence of various concentrations (10 nM - 100 μM) of Paclitaxel in 6-well plates and cultured at 37°C in 5% CO_2 for 24 hr or 48 hr.

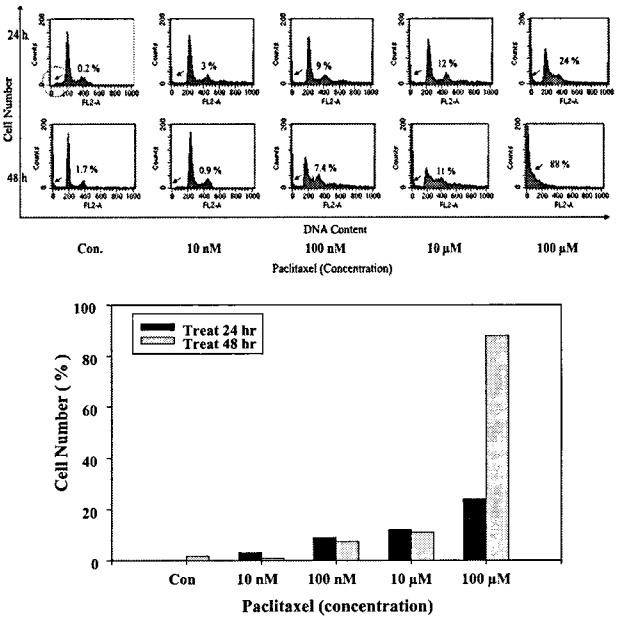

Fig. 5. Paclitaxel regulated Sub-G_1 Arrest in HeLa S_3 Cells. 1 × 10^4 cells were plated in 2 ml growth medium in the presence or absence of various concentrations (10 nM-100 μM) of paclitaxel in 6-well plates and cultured at 37°C in 5% CO_2 for 24 hr or 48 hr. In each panel, flow-cytometric measurements of DNA content after PI staining as described under "Materials and methods".

Measurement of Annexin-V in HeLa S_3 cells

To confirmed apoptosis rate of HeLa S_3 cells by paclitaxel, Annexin- V Flow Cytometric experiments were performed. The change in location of phosphatidyl-serine in the cell membrane during apoptosis can be detected with Annexin-V. Costaining with Annexin V and PI allows differentiation of viable cells (Annexin-V negative, PI-negative) from early apoptotic cells (Annexin V-positive, PI-negative) and late apoptotic cells (Annexin V positive, PI-positive). HeLa S_3 Cells were incubated for 48 hr with various concentrations of paclitaxel (0, 10, 100 nM, 10, 100 μM). As shown in Fig. 6, the percentages of both early (bottom right quadrant) and late (top right quadrant) apoptotic cells increased in a dose-dependent.

Expression of PARP, Bcl-2, and Bax proteins

To determine whether paclitaxel treatment causes activation of PARP in HeLa S_3 Cells, western blotting of the PARP performed that using antibodies capable of recognizing the pro-forms and activated form of PARP. As shown in Fig. 7, treatment of the cells with 10, 100nM and 1, 10, 100 μM paclitaxel for 48hr increased the cleavage of PARP to its 89 kDa active forms in these cells. paclitaxel induced

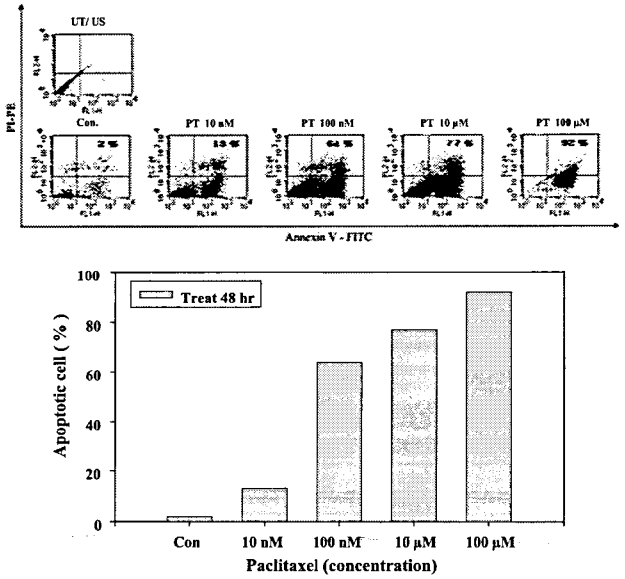

Fig. 6. Measurement of Annexin-V in HeLa S_3 cells. For Annexin-V Binding Assay, 1×10^4 cells were plated in 2 ml growth medium in the presence or absence of various concentrations (10 nM-100 μM) of paclitaxel in 6-well plates and cultured at 37°C in 5% CO_2 for 24 hr or 48 hr. To confirmed apoptosis rate of HeLa S_3 cells by paclitaxel, Annexin- V flow cytometric experiments were performed as described by the manufacturer.

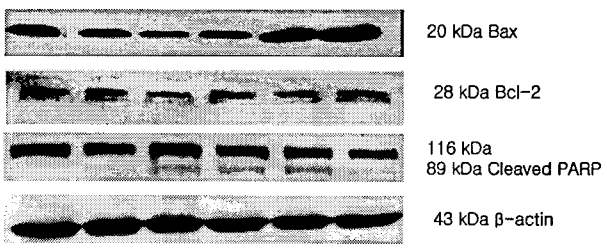

Fig. 7. Western blot analysis of expression of PARP, Bcl-2, Bax proteins in HeLa S_3 cells treated with paclitaxel. Cells treated with paclitaxel showed the decrease of Bcl-2 proteins and the cleavage of PARP.

more cleavage of the well characterized caspase-3 substrate, PARP, relative to individual treatments. Fig. 7 showed Bcl-2 and Bax expression levels. The representative apoptogenic factor Bax level increased, but expression of survival factor Bcl-2 was decreased.

Discussion

Paclitaxel is a highly active anticancer drug that triggers apoptosis in wide spectrum of cancer cells [16,30,42]. The primary mechanism of the action of paclitaxel is attributed to its ability to bind to microtubules and prevent their assembly, causing cells to arrest in the Sub-G_1 phase and thereby blocking cell cycle progression [29]. Although this explains the underlying mechanism of paclitaxel-mediated growth arrest, its efficacy exceeds that of conventional microtubule-disrupting agents, suggesting that additional cellular effects may be operating via pathways independent of mitotic arrest. These results also support this notion. This study is the first to provide experimental evidence demonstrating that a dose-dependent cytotoxic effect by paclitaxel in HeLa S_3 cells. Also, cell viability of the other cancer cells decreased in a dose-dependent by paclitaxel that was observed. And in this experiment demonstrate that paclitaxel induced Sub-G_1 arrest in HeLa S_3 cells in a dose-dependent. Number of cells in S phase decresed, but in Sub-G1 phase increased. Finally, results of this report indicated that paclitaxel not only induced apoptosis, but also induced cell cycle arrest at the sub-G_1 phase in HeLa S_3 cells.

Apoptosis is initially characterized by morphological changes of dying cells [2,7,17]. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by DAPI. This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. They are also visible in permeabilized apoptotic cells stained with other DNA binding dyes like DAPI. That is why, morphological change (fragmentation) by various concentrations of paclitaxel that was observed in this report. This result shows that paclitaxel induced apoptosis through nuclei condensation.

In addition to these changes in cell morphology that occur during apoptosis, a major event is the loss of membrane phospholipid asymmetry, with translocation of phosphatidylserine (PS) from the inner leaflet of the phospholipid bilayer to the cell surface. While the function of PS externalization is unclear, it may serve as a "signal" for recognition by phagocytic cells which respond by engulfing the apoptotic cell before loss of plasma membrane integrity. Exposure of PS on the cell surface provides a simple means for detecting cells undergoing apoptosis. Fluorochrome conjugates of annexin V can be used to monitor changes in cell membrane phospholipid asymmetry, thereby providing a convenient tool for detection of

apoptotic cells [11,41]. Ultimately, this report confirmed that apoptosis rate of HeLa S_3 cells by paclitaxel increased in a dose-dependent manner.

Additionally, Apoptosis induced by different stimuli, such as death ligands, chemotherapeutic drugs, or ionizing irradiation, leads to the activation of caspase [14]. In this report demonstrated that PARP, caspase-3 substrate, were activated during apoptosis induced by various concentrations of paclitaxel. Finally, paclitaxel induced more cleavage of the well characterized caspase-3 substrate, PARP, relative to individual treatments.

Taken together, these results lead to the conclusion that the effect of paclitaxel in inducing apoptosis in HeLa S_3 cells that is dependent cell cycle arrest and PARP activation. In conclusion, all observations indicate that paclitaxel may provide a superior therapeutic index and advantage in the clinic for the treatment of cervical cancer.

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초록 : HeLa S_3 자궁암 세포에서 paclitaxel에 의해 유도된 Poly(ADP-ribose) Polymerase 분철과 세포자멸사와의 관계

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Paclitaxel이 암세포에서 세포예정사를 유발할지라도, 아직 정확한 기전은 잘 알려져 있지 않다. 이에 본 연구에서는 HeLa S_3 자궁암세포에서의 paclitaxel이 어떠한 영향을 미치는지 알아보고자 한다. 그리하여 방법으로는 세포독성검사, apoptotic cells의 형태학적 변화(DAPI 염색), western blot 분석법을 사용하여 수행하였다. 본 연구의 결과로 paclitaxel은 HeLa S_3 세포에서 세포독성을 보이며, 특히 paclitaxel의 IC_{50} 값은 약 1 μM이며, paclitaxel 처리한 HeLa S_3 세포에서 형태학적 변화(분절화)를 관찰하였고, flow cytometric 분석에서는 G2/M기가 차단되어 paclitaxel은 세포주기 특히 Sub-G_1기를 조절함을 알 수 있다. 그리고Paclitaxel을 처리한 HeLa S_3 세포에서는 PARP cleavage를 유발하였고 Bcl-2의 감소와도 관련되었다.