ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2003) 21: 31-41

Cytotoxicity and Apoptosis of Ovarian and Breast Cancer Cell Lines Induced by OVS1 Monoclonal Antibody and Paclitaxel

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SUMMARY **OVS1 monoclonal antibody (MAb) produced against ovarian cancer is currently used to identify mucinous cystadenocarcinoma antigen as a tumor marker secreted in serum. The potential of OVS1 MAb in ovarian cancer treatment was studied by evaluating the induction of cytotoxicity and apoptosis of SKOV3 ovarian cancer and BT549 breast cancer cell lines induced by OVS1. Paclitaxel, an antitumor drug, was used as positive control and applied as a combined drug together with OVS1 MAb. OVS1 MAb and paclitaxel were found by MTT assay to induce cytotoxicity against both cell lines. The ED_{50} of OVS1 MAb were 26.25 and 25.00 μg/ml and of paclitaxel were 21.88 and 9.20 nM against SKOV3 and BT549 cell lines, respectively. The quantitative amount of cells determined by fluorimetric assay was correlated to the results of the MTT assay. The combined application of OVS1 MAb and paclitaxel on these two cell lines resulted in a greater cytotoxicity than observed by either agent alone. OVS1 MAb and paclitaxel applied against both cell lines induced the morphological changes of apoptotic cell death at 24 hours visualized by two color fluorescence dyes, Ho33342 and propidium iodide. Combination of the two substances enhanced the rate of apoptosis compared to either OVS1 MAb or paclitaxel given alone. DNA fragmentation was detected in an agarose gel electrophoresis after treating cells with OVS1 MAb and paclitaxel at 24 hours. These findings on the induction of cytotoxicity and apoptosis by OVS1 MAb on cancer cell lines have implications on the potential application of OVS1 MAb for clinical therapy.**

Death from cancer is still increasing each year in spite of modern methods of treatment.[1] Cervical, breast, oral cavity, lung and ovarian cancers are the major cancers prevalent in women around the world.[2] Ovarian cancer is ranking fifth and breast cancer is second regarding incidence of female malignancies in Thailand. However, ovarian cancer is the leading cause of death from gynecologic malignancies, since the tumor is often in an advanced stage by the time of clinical diagnosis.[3]

For several decades, tumor immunology has been the focus of research for improved diagnosis and treatment of cancers.[4] Advanced knowledge improved the protocols of treatment for many cancers and the survival rates of patients became up to 5 years and longer. Monoclonal antibody could become one of the major tools to achieve an improved outcome.[5] Monoclonal antibodies with high sensitivity and specificity for ovarian cancer are still required for diagnosis and treatment, especially in the early stages of ovarian and breast cancers.

Mucinous cystadenocarcinoma is prevalent in Thailand.[7] Neungton *et al.*[6] reported the production of OVS1 MAb by fusing a murine myeloma cell line with

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spleen cells from mice immunized with fresh human ovarian mucinous cystadenocarcinoma cells. OVS1 MAb was selected by immunohistochemistry and showed 96% specificity and 67% sensitivity to mucinous cystadenocarcinoma without cross reaction to normal cells, i.e. benign or non-ovarian cancer tissue. The result confirmed the highly specific and moderately sensitive recognition of OVS1 antigen presented on cancer tissue. OVS1 MAb could determine specific antigen levels in the serum and identify them as tumor markers. This improved diagnosis has been reported to be beneficial to cancer patients.

Apart from the benefit of OVS1 MAb for diagnosis, the application of OVS1 MAb for cancer treatment was studied. The cytotoxic and apoptotic activities of OVS1 MAb against ovarian and breast cancer were investigated. Paclitaxel, a potent anticancer drug, was used as positive control. It is a complex diterpene, isolated from the bark of the Western *Taxus brevifolia*, and has been approved by the US Food and Drug Administration for use in refractory ovarian cancer. Paclitaxel is a potent inhibitor of cell proliferation that arrests cells in mitosis and induces multinucleation of cells in the interphase.[7-13] Following its introduction into clinical trials, the drug was approved for treatment of ovarian cancer and also has promising activities against cancer of breast, lung, esophagus, head and neck. Paclitaxel may benefit advanced ovarian cancer patients who have become resistant to standard chemotherapy.[14]

In this study, combined exposure of OVS1 MAb and paclitaxel was done to investigate whether OVS1 MAb and paclitaxel would have synergistic effects to induce cytotoxicity and apoptosis in ovarian and breast cancer cell lines. This could lead to further exciting studies effecting clinical strategy in cancer chemotherapy.

MATERIALS AND METHODS

Purification of OVS1 monoclonal antibody

OVS1 MAb was obtained from OVS1 hybridoma cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 10 mM glucose, 1.2 mM pyruvate and 0.7 mM glutamine (GIBCO, Gaithersburg, USA). The culture supernatant containing OVS1 MAb was centrifuged to remove the cell pellet and filtered through a 0.2 μm-pore membrane. The supernatant was gently loaded onto a Protein-A Sepharose affinity chromatography (Pharmacia, Uppsala, Sweden) preequilibrated with 0.05 M phosphate buffered saline (PBS) at pH 8.0. Unbound proteins were removed by washing the column with 0.05 M PBS pH 8.0 and the bound OVS1 MAb was eluted out by using 0.1 M sodium citrate pH 3.0. Each fraction was collected into a tube containing 100 μl 2 M Tris-HCl pH 9.0 so that the final pH of the eluant would become neutral eventually. The peak fractions were pooled, dialyzed against 0.05 M PBS pH 7.4 and concentrated further by a SpeedVac concentrator (Savant, New York, USA). The purified OVS1 MAb was quantitated by a MicroBCA protein assay (Pierce, Illinois, USA) and stored at -80°C before further use.

Detection of OVS1 MAb using a double sandwich ELISA

OVS2 MAb, another MAb

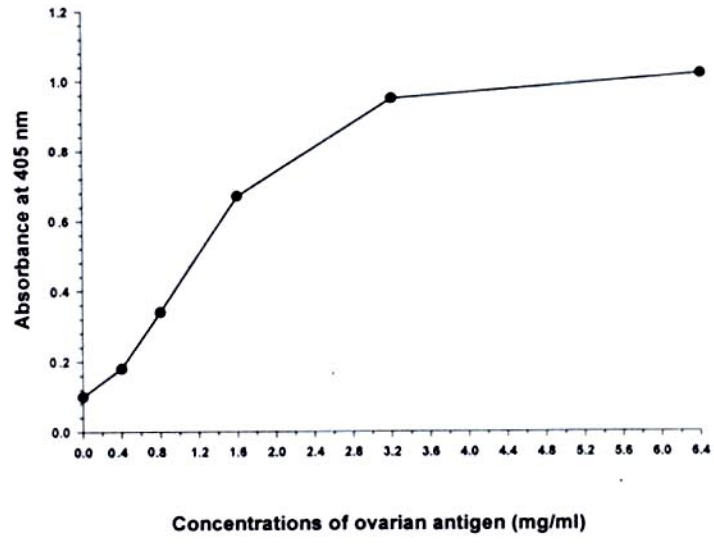

Fig. 1 Dose response curve of OVS1 MAb and various concentrations of ovarian antigen by double sandwich ELISA technique.

recognizing a different epitope on ovarian cancer cells was purified by the same method as OVS1 MAb and labeled with biotin. Ovarian antigen obtained from ovarian tumor cells was prepared as described by Neungton *et al.*[15] one hundred microliters of OVS1 MAb (5 μg/ml) was immobilized on the surface of a microtiter plate and incubated at 4°C overnight. Each well was washed with 0.1% Tween-20 in PBS, added with 200 μl 3% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, USA) and incubated for 2 hours at 37°C. The wells were washed again and serial concentrations of ovarian antigen were added and incubated at 37°C for 1 hour. After washing, 100 μl of OVS2 MAb-biotin was added into each well, incubated for 1 hour after which the excessive OVS2 MAb was washed out. One hundred microliters per well of 1 μg/ml of streptavidin-alkaline phosphatase (Sigma) was added and incubated at 37°C for 15 minutes. Then 100 μl per well of 1,000 μg/ml *p*-nitrophenyl phosphate substrate (Sigma) was added in each well and incubated at 37°C for 30 minutes in the dark. The reaction was terminated by adding 50 μl of 2.5 N sulfuric acid and the color reaction was measured at 405 nm with a microplate reader (Molecular Devices, California, USA).

Cell proliferation assay

SKOV3 human ovarian and BT549 human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI-1640 medium. Serial dilutions of samples (50 μl) and cells ($1x10^4$ cells/50 μl) were added into each well of the 96-well plates and incubated for 48 hours at 37°C in a 5% CO_2 incubator. The medium was removed and cells were further incubated with Hanks' balanced salt solution (HBSS) (Sigma) containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) for 2 hours. The MTT -solution was then discarded and 50 μl of isopropanol (Carlo Erba, Milano, Italy) was added into each well to solubilize the cells and dissolve the color substance. The plates were then vigorously agitated for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative from

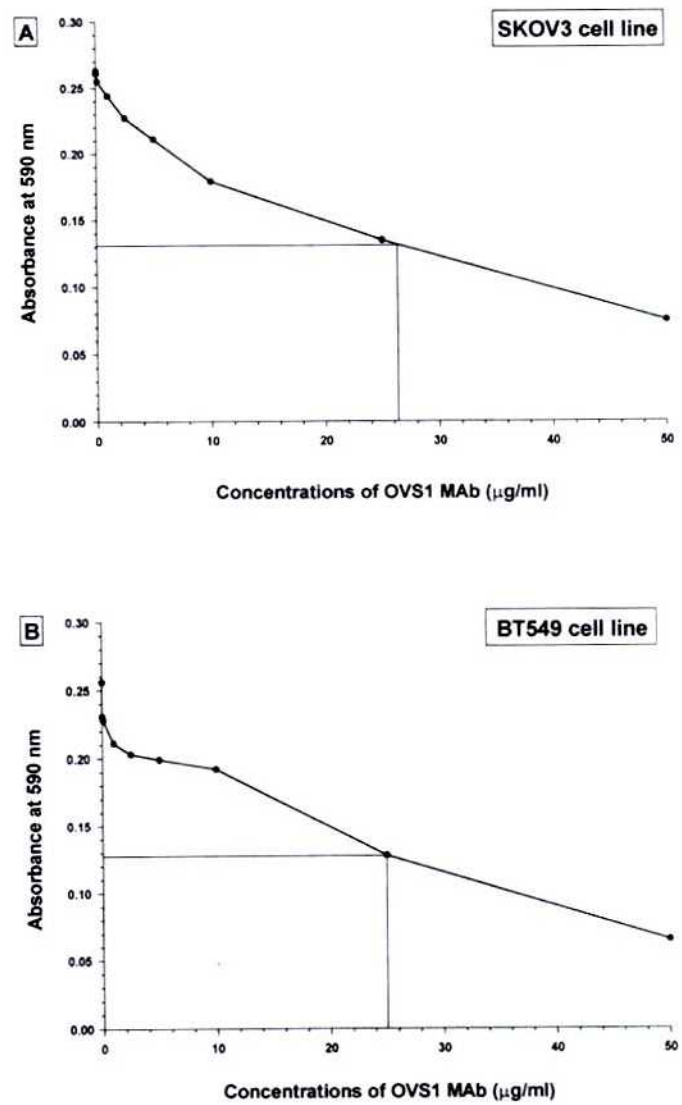

Fig. 2 MTT cytotoxicity assay of OVS1 MAb against SKOV3 (A) and BT549 (B) cells. OVS1 MAb concentrations varied from 0.01 to 50 μg/ml. The ED_{50} of OVS1 MAb on SKOV3 (A) and BT549 (B) cells were 26.25 and 25.00 μg/ml, respectively.

the MTT was analyzed with a microplate reader at 590 nm.[16,17]

Two-color fluorescent DNA staining

The morphological changes of apoptotic cell death can be observed by this method. Cells were plated at a density of 5 x 10^5 cells and grown in a 35 x 10 mm cell culture dish for 24 hours. After incubation, cells were treated with samples at various incubation times and the medium was removed after incubation. Benzimidazole Hoechst 33342 (Ho33342) (Sigma) at 1 μg/ml and propidium iodide (PI) (Sigma) at 5 μg/ml were concomitantly added to the cells and incubated at 37°C for 15 minutes. The cells were washed once with cold HBSS before counting and observation of morphological changes with a phase contrast-fluorescence inverted microscope (Zeiss, Germany).[17,18]

The agarose DNA ladder assay

Cells (3 x 10^6 cells) were grown in a culture flask for 48 hours and treated with sample. After removing the medium, the cells were gently harvested by centrifugation at 5,000 x *g* for 5 minutes. The cell pellets were incubated with 100 μl of lysis buffer (100 mM Tris-HCl pH 8, 100 mM NaCl and 10 mM ethylenediamine tetraacetic acid [Sigma]) for 60 minutes at 50°C. Ten microliters of 20 mg/ml proteinase-K (Sigma) was added onto the cells and further incubated at 50°C overnight. RNase (3 μl of 10 mg/ml) was then added and incubated for 2 hours at 50°C after which the DNA was extracted with phenol-chloroform-isoamyl alcohol (Carlo Erba) as described by Studzinski[17] and Yang *et al.*[19] The extracted DNA was applied to a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under a UV light transilluminator (Fotodyne, Wisconsin, USA).

RESULTS

Purification of OVS1 monoclonal antibody

OVS1 MAb was purified from the supernatant by Protein-A Sepharose 4B affinity chromatography. The absorbance of each fraction was measured at 280 nm and the fractions with a high optical density were pooled and dialyzed against 0.05 M PBS pH 7.4. The total amount of OVS1 MAb obtained was between 1.32-2.34 mg from 310-630 ml of supernatant.

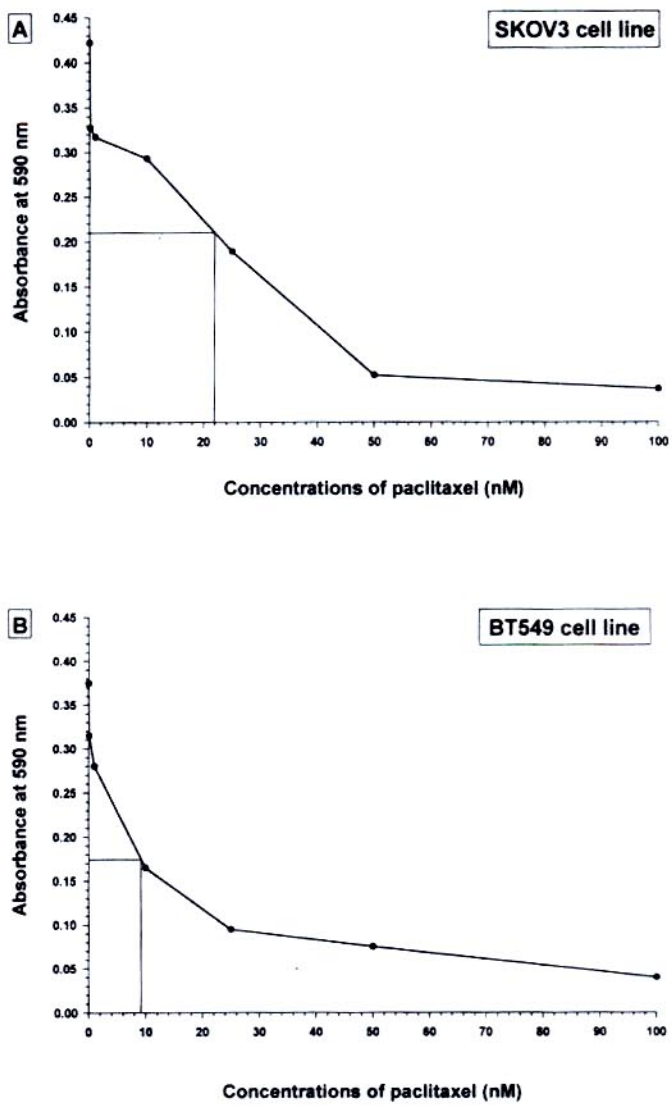

Fig. 3 MTT cytotoxicity assay of paclitaxel against SKOV3 (A) and BT549 (B) cells. Paclitaxel concentrations varied from 0.01 to 100 nM. The ED_{50} of paclitaxel on SKOV3 (A) and BT549 (B) cells were 21.88 and 9.20 nM, respectively.

Detection of OVS1 MAb by double sandwich ELISA

This experiment was performed to identify the specificity of purified OVS1 MAb against ovarian antigen. One hundred microliters of OVS1 MAb (5 μg/ml) was adsorbed onto each well and ovarian antigen was diluted between 0.2 to 6.2 mg/ml. MAb against typhoid antigen (kindly provided by Prof. Dr. Suthipan Sarasombath, Siriraj Hospital) was used as negative control. The absorbances increased in an ovarian antigen dose-dependent fashion became and stable when the concentration of antigen was higher than 3.2 mg/ml (Fig. 1). It showed that OVS1 MAb was specific to ovarian antigen with no cross-reaction to other antigens (normal ovarian and normal serum antigens) corresponding to the report of Neungton *et al.*[15]

Cell proliferation assay

The cytotoxic effects of OVS1 MAb, paclitaxel, as well as the combination of OVS1 MAb and paclitaxel on SKOV3 and BT549 cell lines were investigated by the MTT method. OVS1 MAb can significantly inhibit the proliferation of SKOV3 and BT549 cell lines. The ED_{50} were 26.25 and 25.00 μg/ml on SKOV3 and BT549 cells, respectively (Fig. 2). Paclitaxel was found to inhibit the proliferation of both kinds of cells in the same dose-dependent manner as OVS1 MAb, with an ED_{50} at 21.88 and 9.20 nM on SKOV3 and BT549 cells, respectively (Fig. 3). Further studies showed that the combined treatment of OVS1 MAb and paclitaxel enhanced the cytotoxic effect on both cell lines compared with OVS1 MAb or paclitaxel alone (Fig. 4).

Two-color fluorescent DNA staining for detection of apoptosis

After applying OVS1 MAb (25 μg/ml) and paclitaxel (25 nM) on SKOV3 and BT549 cells, the cells were stained with fluorescent dyes and counted using a fluorescent inverted microscope. Ho33342, a membrane-permeant dye, was used to stain DNA of living cells (blue color) and early apoptotic cells (light blue color), whereas PI, a membrane-impermeant dye, stained DNA of late apoptotic and necrotic cells (red color). The results showed the quantity of apoptotic cells increasing in a time-dependent manner (Tables 1-2, Figs. 5-6). Combined exposure of OVS1 MAb and paclitaxel on both cells can enhance the quantity of apoptotic cells re-

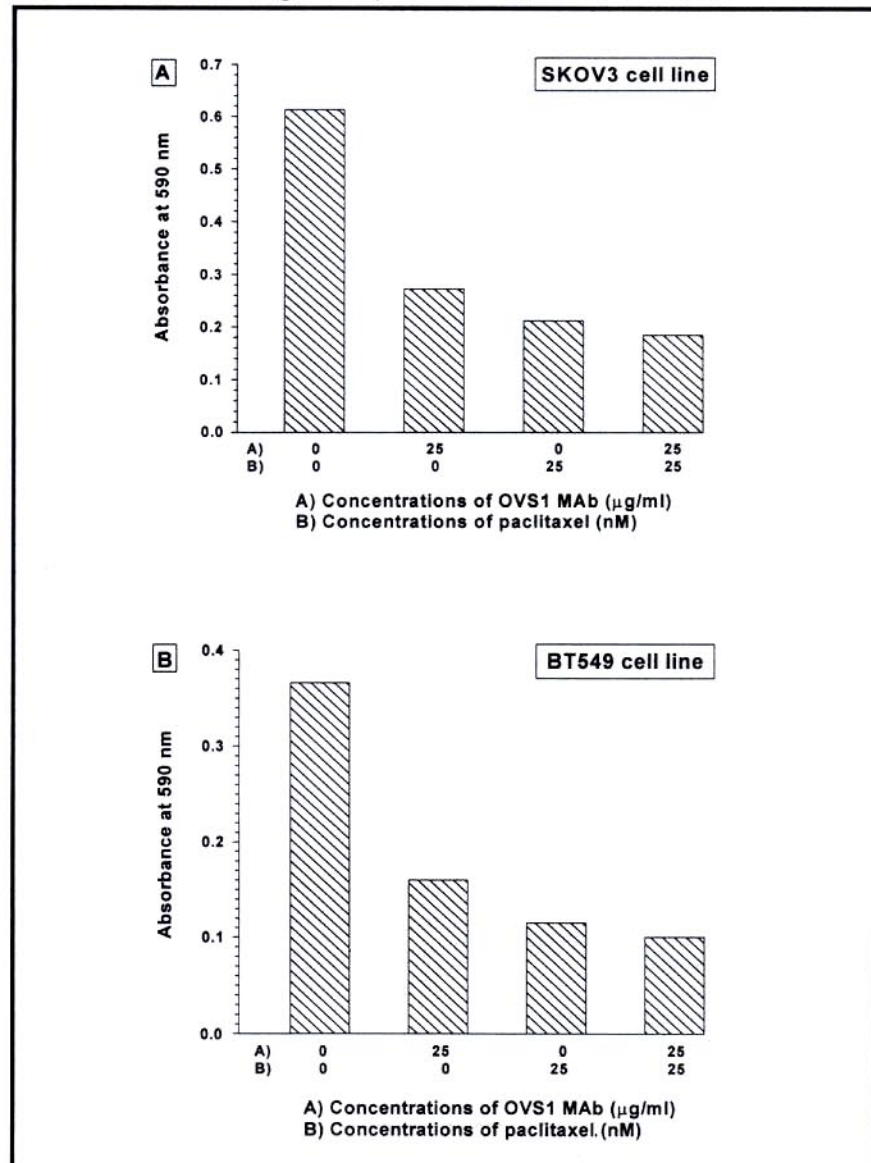

Fig. 4 MTT cytotoxicity assay of combined 25 μg/ml OVS1 MAb and 25nM paclitaxel against SKOV3 (A) and BT549 (B) cells.

Table 1 Determination of apoptosis of SKOV3 cells by two-color fluorescent DNA staining after treating with OVS1 MAb and paclitaxel

SKOV3 cells	Incubation time	Percentage of cell types (%)*			
		Living	Apoptotic	Late apoptotic and necrotic	Total apoptotic and necrotic
Control	24 h.	94.07	3.56	2.37	5.93
Treated with 25 μg/ml OVS1 MAb	16 h.	81.40	16.12	2.48	18.60
	24 h.	77.10	18.69	4.21	2.90
	48 h.	63.18	14.93	21.89	36.82
Treated with 25 nM paclitaxel	16 h.	77.55	20.18	2.27	22.45
	24 h.	50.44	38.75	10.81	49.56
	48 h.	56.20	14.87	28.93	43.80
Treated with combined 25 μg/ml OVS1 MAb and 25 nM paclitaxel	24 h.	43.23	50.00	6.77	56.77

*Total cell counts at 200 cells

Table 2 Determination of apoptosis of BT549 cells by two-color fluorescent DNA staining after treating with OVS1 MAb and paclitaxel

BT549 cells	Incubation time	Percentage of cell types (%)*			
		Living	Apoptotic	Late apoptotic and necrotic	Total apoptotic and necrotic
Control	24 h.	96.40	3.27	0.33	3.60
Treated with 25 μg/ml OVS1 MAb	16 h.	87.34	10.97	1.69	12.66
	24 h.	72.33	21.36	6.31	27.67
	48 h.	76.69	10.12	13.19	23.31
Treated with 25 nM paclitaxel	16 h.	87.50	11.64	0.86	12.50
	24 h.	80.49	18.18	1.33	19.51
	48 h.	79.78	7.98	12.24	20.22
Treated with combined 25 μg/ml OVS1 MAb and 25 nM paclitaxel	24 h.	48.30	35.27	16.43	51.70

*Total cell counts at 200 cells

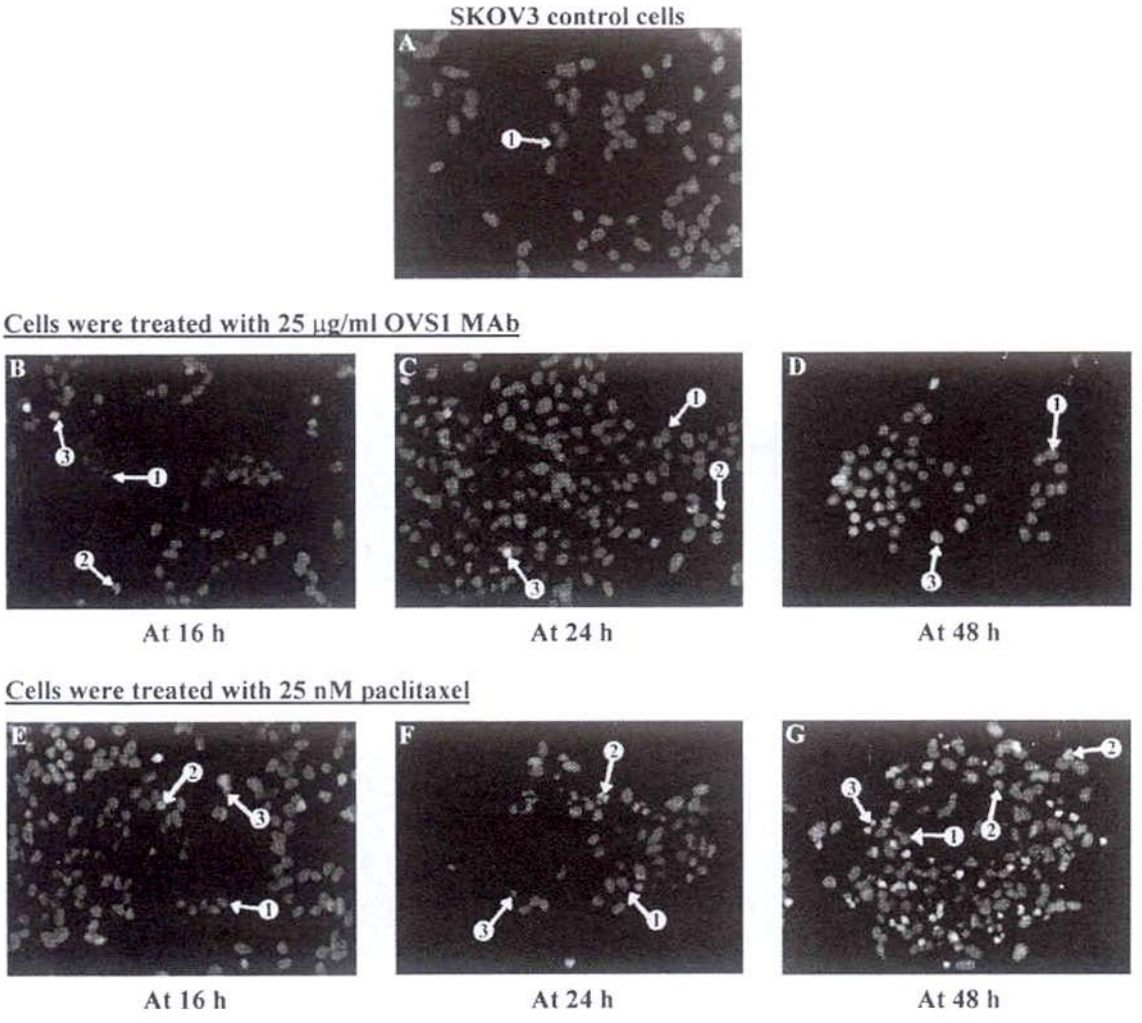

Fig. 5 Morphological changes of SKOV3 cells after treatment with OVS1 MAb and paclitaxel at various incubation times. **A**: SKOV3 control cells; **B, C, D**: cells were treated with 25 μg/ml OVS1 MAb for 16, 24 and 48 hours, respectively; **E, F, G**: cells were treated with 25 nM paclitaxel for 16, 24 and 48 hours, respectively. ①: Living cells, stained blue with a consistent color and normal cell size; ②: Apoptotic cells, stained light blue with a smaller cell size and sometimes fragmented nucleus; ③: Late apoptotic and necrotic cells, stained red with small and round nucleus or swollen nucleus.

markably (Tables 1-2), similar to their combined cytotoxic effect.

After treating SKOV3 cells for 24 hours with 10 μg/ml of OVS1 MAb, observation on morphological changes by phase contrast-fluorescence inverted microscope at 400x magnification, showed the characteristic patterns of apoptotic cell death, including apoptotic bodies, nuclear fragmentation, nuclear shrinkage and membrane blebbing (Fig. 7).

DNA fragmentation by agarose gel electrophoresis

This method is a biochemi-

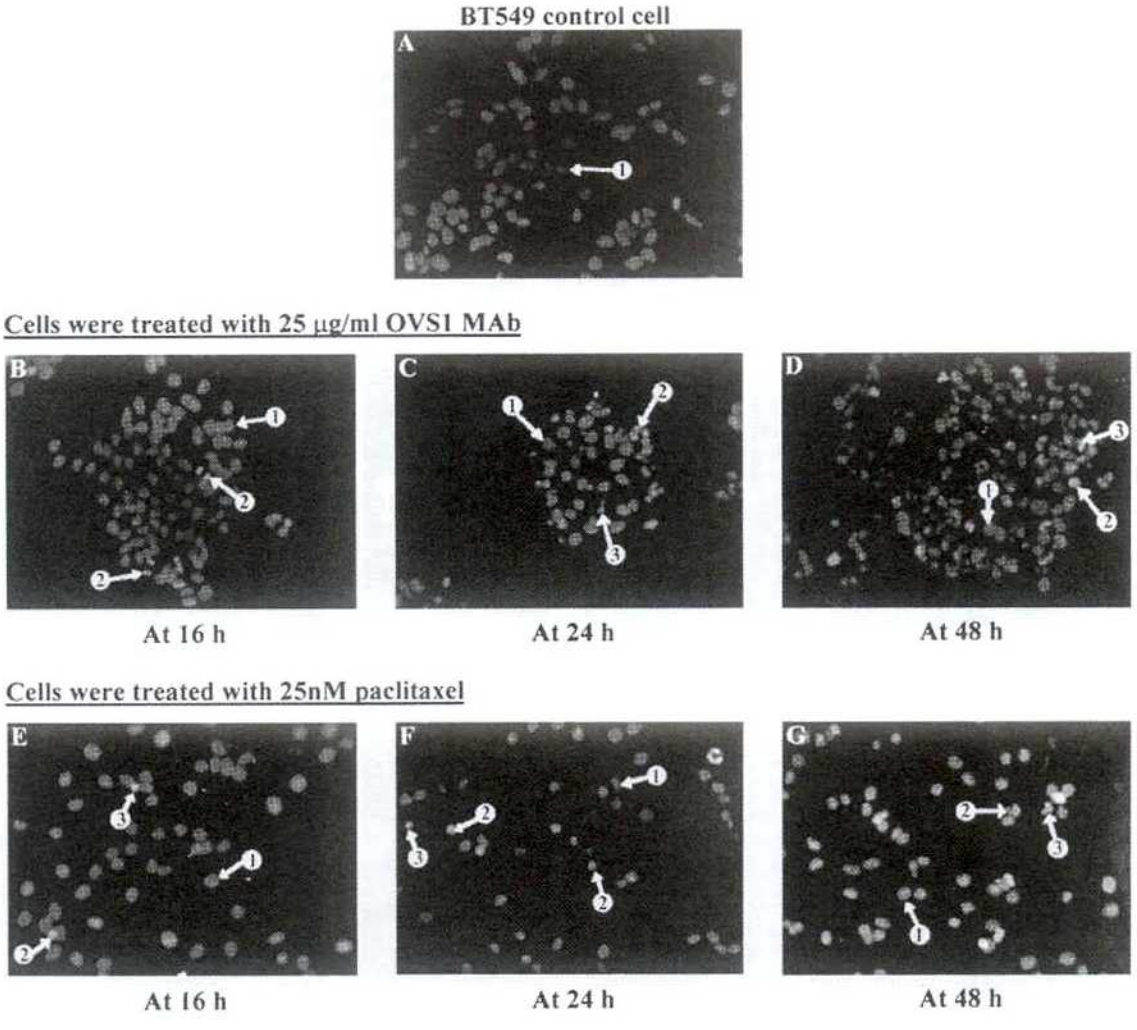

Fig. 6 Morphological changes of BT549 cells after treatment with OVS1 MAb and paclitaxel at various incubation times. **A**: BT549 control cells; **B**, **C**, **D**: cells were treated with 25 μg/ml OVS1 MAb for 16, 24 and 48 hours, respectively; **E**, **F**, **G**: cells were treated with 25 nM paclitaxel for 16, 24 and 48 hours, respectively; ①: Living cells, stained blue with a consistent color and normal cell size; ②: Apoptotic cells, stained light blue with a smaller cell size and sometimes fragmented nucleus; ③: Late apoptotic and necrotic cells, stained red with a small and round nucleus or swollen nucleus.

cal technique which is often used to confirm apoptotic cell death. DNA ladders of SKOV3 cells were tested by a 1.5% agarose gel electrophoresis after treatment with 25 μg/ml of OVS1 MAb and/or 25 nM of paclitaxel for 24 hours and compared to untreated control cells (Fig. 8). The results revealed that OVS1 MAb and paclitaxel induced apoptosis of SKOV3 cells at the above-mentioned doses and incubation times.

DISCUSSION

The present study was designed to compare the cytotoxic and apoptotic activities of OVS1 MAb, paclitaxel and the combined

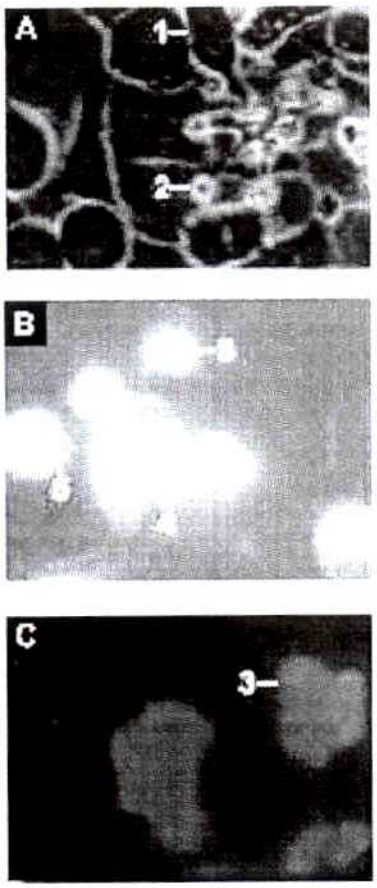

Fig. 7 Morphological changes of SKOV3 cells following exposure to 10 μg/ml of OVS1 MAb. **A**: Cells were observed under phase contrast microscope; **B**: Cells were stained by Ho33342 and examined under a fluorescence inverted microscope; **C**: Cells were stained by PI and examined under a fluorescence inverted microscope. **1**: Normal cells; **2**: Apoptotic bodies; **3**: Nuclear fragmentation; **4**: Nuclear shrinkage; **5**: Membrane blebbing.

treatment of OVS1 MAb and paclitaxel on SKOV3 ovarian and BT549 breast cancer cell lines. The *in vitro* cytotoxicity assays of these substances were evaluated by the MTT method which living cells can only reduce MTT to insoluble formazan crystal. OVS1 MAb was purified from the culture medium of OVS1 hybridoma. OVS1 MAb showed very strong inhibition on the proliferation of SKOV3 and BT549 cell lines in a dose-dependent fashion. The ED_{50} of OVS1 MAb on SKOV3 and BT549 cells were found to be 26.25 and 25.00 μg/ml, respectively. Paclitaxel is one of the most important anticancer drugs developed in the past two decades. It is a powerful inhibitor of cell proliferation and arrests cells in mitosis and induces multinucleation of cells in interphase. Despite the considerable knowledge gained in the past several years, the precise molecular cytotoxic mechanisms of paclitaxel have not yet been conclusively established. This study showed that paclitaxel has potent cytotoxic effects on both cell lines investigated with an ED_{50} by MTT assay of 21.88 and 9.20 nM for SKOV3 and BT549 cells, respectively. Notably, combined application of OVS1 MAb and paclitaxel can enhance their cytotoxic activity on both cell lines compared to either OVS1 MAb or paclitaxel given alone. This finding of an enhanced cytotoxic activity could be due to different mechanisms of action of these two substances.

Subsequent experiments showed that both OVS1 MAb and paclitaxel clearly induced apoptosis in SKOV3 and BT549 cells. The induction of apoptosis was studied by two-color fluorescent DNA staining and agarose gel electrophoresis. Two-color fluorescent DNA staining has been used to identify apoptotic and necrotic sub-populations. Ho33342 (blue color) visualized early apoptotic cells, which exhibited classic nuclear alterations such as condensation and fragmentation of DNA. Whereas, late apoptotic and necrotic cells were stained with PI (red color), which showed swollen and round nuclei without any nuclear condensation and fragmentation.[17,18] Using this technique, the results showed that apoptotic cell death remarkably increased in a time-dependent manner after treatment with OVS1 MAb (25 μg/ml) and paclitaxel (25 nM). Viable cells were gradually reduced with in-

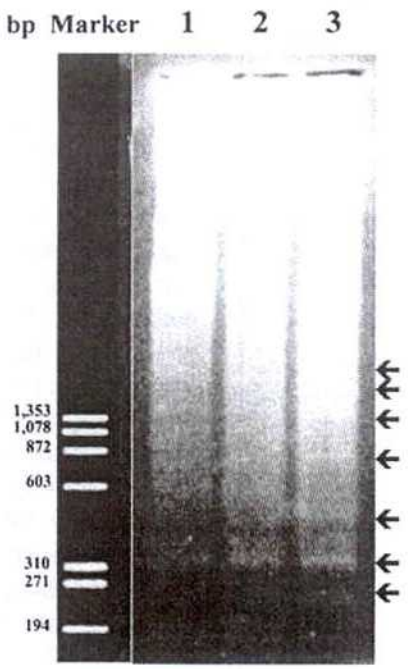

Fig. 8 Analysis of DNA fragmentation in 1.5% agarose gel electrophoresis after treating SKOV3 cells with OVS1 MAb and paclitaxel. Lane 1: SKOV3 control cells; Lane 2: Cells treated with 25 nM paclitaxel for 24 hours; Lane 3: Cells treated with 25 μg/ml OVS1 MAb for 24 hours; ← : Ladder of DNA.

creased incubation time. Moreover, a combined treatment of OVS1 MAb and paclitaxel increased the numbers of apoptotic cells compared to either OVS1 MAb or paclitaxel alone at 24 hours. In addition, we could demonstrate morphological changes in the cancer cells after treatment with 10 μg/ml OVS1 MAb using 400x magnification, including apoptotic bodies, nuclear fragmentation, nuclear shrinkage and membrane blebbing (Fig. 7). Oligonucleosomal DNA fragments (ladders) of cells were demonstrated as another hallmark of apoptosis by 1.5% agarose gel electrophoresis.[17] The fragmentation of DNA was substantial after exposing SKOV3 cells to 25 μg/ml of OVS1 MAb and 25 nM of paclitaxel for 24 hours. However, DNA ladders are difficult to demonstrate and by itself insufficient to justify the description of the phenomenon as apoptosis, since the apoptotic process can occur without DNA ladders.[17] In some non-lymphoidal cells no typical nucleosomal DNA cleavage was observed.[20]

In conclusion, OVS1 MAb and paclitaxel can induce cytotoxicity and apoptosis on ovarian and breast cancers. Moreover, combined treatment enhanced cytotoxic and apoptotic activities more than given alone which was also confirmed by the experiments performed later by FACS (data not shown). This is the first report of a therapeutic strategy that used a combination of paclitaxel and OVS1 MAb. Since OVS1 MAb has a greater specificity and sensitivity to mucinous cystadenocarcinoma which is the most prevalent ovarian cancer in Thailand.[15] The results from this trial will shed further light on the clinical utility approach. The ultimate goal of applying antibody combined to antitumor drug or other medicinal plant constituents discovered in the future will be beneficial to cancer therapy.

ACKNOWLEDGEMENTS

This work was supported by grants from the Faculty of Graduate Studies and Faculty of Pharmacy, Mahidol University and the Government Pharmaceutical Organization.

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