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European Journal of Cancer 37 (2001) 531–541

European Journal of Cancer

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Combination effect of adenovirus-mediated pro-apoptotic *bax* gene transfer with cisplatin or paclitaxel treatment in ovarian cancer cell lines

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Received 11 July 2000; received in revised form 28 September 2000; accepted 28 September 2000

Abstract

To develop a novel therapeutic strategy for ovarian cancer, we constructed a recombinant adenovirus which highly expresses pro-apoptotic Bax protein and examined its therapeutic effect on a series of ovarian cancer cell lines: A2780, A2780/cDDP, OVCAR-3 and SK-OV-3. A recombinant adenovirus carrying the *Bax-α* gene (AxCALNKYbax) induced high expression of the Bax-α protein in all the cell lines. The cytotoxic effect of Bax was observed in three ovarian cancer cell lines: the per cent reduction in the number of cells was 40.0% for cisplatin-sensitive A2780, 50.0% for cisplatin-resistant A2780/cDDP, and 64.8% for marginally cisplatin-resistant OVCAR-3. In contrast, it was only 12.3% for cisplatin-resistant SK-OV-3. Cisplatin-resistant A2780/cDDP had a *p53* mutation and exhibited attenuated Bax induction after cisplatin treatment, which may explain why supplementation of Bax was effective in this chemoresistant ovarian cancer. Combination with cisplatin or paclitaxel enhanced the cytotoxic effect of Bax induction in all but one cell line including cisplatin-resistant A2780/cDDP. It appears that adenovirus-mediated Bax induction, with or without combination with conventional chemotherapy, useful strategy for the treatment of ovarian cancer.

Keywords: Apoptosis; Bax; Ovarian cancer

1. Introduction

Ovarian cancer is one of the most lethal malignancies in women [1,2]. Since more than two-thirds of patients with ovarian cancer are in the advanced stage at the time of diagnosis, adjuvant chemotherapy following debulking surgery is important for successful treatment [1]. Although the majority of ovarian cancers are relatively sensitive to initial chemotherapy, they readily acquire drug resistance during the course of treatment. Over the past three decades, the 5-year survival rate has shown little improvement despite the use of cisplatin [3]. To overcome this situation, novel therapeutic strategies, including gene therapy, are urgently needed.

Recent research on the biology of cell death has revealed that the cytotoxic effects of various antitumour reagents are mediated by apoptosis, and that chemotherapy is effective only when the apoptotic pathway in the tumour cells remains intact [4]. If this pathway is disturbed or impaired, tumour cells acquire drug resistance. The apoptotic pathway is strictly regulated by a number of genes and gene products such as p53, the Bcl-2 family, Fas and insulin-like growth factor (IGF)-1 [5–7]. Alteration of these genes has been shown to be the first event in a process leading to drug resistance [7]. For instance, *p53* mutation, one of the most common genetic events related to ovarian cancer, has been implicated in chemoresistance in clinical ovarian cancer [8] as well as in *in vitro* experiments [9]. Similarly, the altered expression of members of the Bcl-2 family has been shown to be associated with sensitivity to cisplatin [10]. Therefore, if apoptosis-related genes could be controlled in ovarian cancer, this might provide a useful

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0959-8049/01/$ - see front matter
PII: S0959-8049(00)00431-7

strategy for overcoming drug resistance or for establishing an alternative therapeutic method to chemotherapy.

In this study, we focused on the *Bax* gene, which is well known for promoting apoptosis [11]. In some previous studies, a stably transfected *Bax* gene efficiently induced apoptosis in human cancer cell lines such as MCF-7 and R30C breast cancers [12,13] and SW626 ovarian cancer [4]. This suggests that the *Bax* gene is a potential candidate for cancer gene therapy. As plasmid-mediated gene transfer has the drawback of low transfection efficiency, we sought to develop a suitable method for efficiently inducing the *Bax* gene in ovarian cancer cells which could be used *in vivo*. Adenovirus-mediated gene transfer is a highly efficient gene transfer system which is already in clinical use [14]. In this study, we examined the cytotoxic effects of adenovirus-mediated *Bax* gene transfer in a panel of ovarian cancer cell lines with different cisplatin sensitivities, and compared the biological aspects of Bax induction among these cells. Very recently Tai and colleagues reported the cytotoxic effect of adenovirus-mediated *Bax* gene induction in several breast and ovarian cancer cells [15]. Other aims of our study were the quantitative analysis of apoptosis and investigation of the intracellular apoptotic signal caused by Bax induction or anticancer drugs. We also examined the enhanced cytotoxicity of Bax induction when it was combined with anticancer drugs cisplatin or paclitaxel.

2. Materials and methods

2.1. Cell culture

The human ovarian cancer cell lines SK-OV-3 and OVCAR-3 were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) media supplemented with 10% fetal calf serum (FCS), respectively. The human ovarian cancer cell lines A2780 and A2780/cDDP (a cisplatin-resistant cell line derived from A2780) were provided by Dr Takashi Tsuruo (Cancer Chemotherapy Center, Tokyo, Japan) with the permission of Dr. Thomas C. Hamilton (Fox Chase Cancer Institute, Philadelphia, PA, USA), and were maintained in RPMI containing 10% FCS.

2.2. Molecular analysis of the p53 gene

Genomic DNA was extracted using the standard phenol/chloroform extraction method. Mutations in exons 5–8 of the *p53* gene were screened by polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) as previously described [16]. Primers for exon 5 were 5′-CTGACTTTCAACTCTG-3′ and 5′-AGCCCTGTCGTCTCT-3′; for exon 6, 5′-CTCTGATTCCTCACTG-3′ and 5′-CCAGAGACCC-CAGTTGCAAACC-3′; for exon 7, 5′-TGCTTGCCA-CAGGTCT-3′ and 5′-ACAGCAGGCCAGTGT-3′; and for exon 8, 5′-AGGACCTGATTTCCTTAC-3′ and 5′-TCTGAGGCATAACTGC-3′. The bands from the PCR/SSCP gels were cut out, extracted using EASY-TRAP (TaKaRa, Otsu, Japan), immersed in 10 μl of Tris–ethylene diamine tetra acetic acid (EDTA) buffer, and directly sequenced using Big Dye Terminator Cycle Sequencing Kits and an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Cetus, Northwalk, CT, USA).

2.3. Drug sensitivity assay

The WST-1 assay (modified method of 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT) assay using Premix WST-1, TaKaRa, Otsu, Japan) was used to evaluate quantitatively the chemosensitivity of the cell lines to cisplatin. Briefly, cells were harvested into 96-well (1000 cells/well) tissue culture plates. After overnight pre-incubation at 37°C, serial dilutions of cisplatin were added to quadruplicate wells, and the cells were exposed to the drug for an additional 2 days. Absorbance measurements were performed according to the manufacturer's protocol. The IC 50 values, defined as the cisplatin concentration that reduced the absorbance by 50%, were estimated graphically from the concentration–response curves.

2.4. Construction of recombinant adenovirus expressing the Bax-alpha gene

Recombinant replication-deficient adenovirus was constructed according to a protocol previously published [17]. Briefly, cDNA of the *Bax-α* coding region (a gift from Dr Stanley J. Korsmeyer, Howard Hughes Medical Institute, St. Louis, MO, USA) was blunt-ended and ligated into the *SwaI* site of pAxCALNLw (provided by Dr Izumu Saito, Institute of Medical Science, University of Tokyo, Tokyo, Japan). This cosmid vector is driven by the CAG promoter and bears an on/off-switching unit which is activated by excisional deletion of an interposed stuffer DNA by the Cre recombinase [18,19]. The hemagglutinin (HA; YPYDVPDYA) epitope sequences were originally inserted into the 5′ site of the *Bax-alpha* gene. The direction and sequence of the insert were confirmed by sequencing analysis. The constructed cosmid DNA was mixed with DNA-terminal protein complex of Ad5-dlX which had been digested with *EcoT22I*, and co-transfected into 293 cells using the calcium phosphate precipitation method (using a CellPhect Transfection Kit, Amersham Pharmacia Biotech, Tokyo, Japan) [16]. The resulting homologous recombination produced a

recombinant adenovirus designated AxCALNKYbax. The Cre recombinase-expressing adenovirus AxCANcre was purchased from Riken DNA Bank (Tsukuba, Japan). Ax1w1, a control recombinant adenovirus that carries no transgene and AxCA*LacZ*, encoding the beta-galactosidase gene, were provided by Dr Izumu Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan). All the recombinant adenoviruses were purified, titered using a standard protocol [16] and used for subsequent experiments.

2.5. Transduction efficiency of adenovirus-mediated LacZ gene

Cells plated in 6-well plates (2×10^4 cells/well) were incubated with various amounts (0–1000 plaque-forming units (pfu)/cell) of AxCA*LacZ* for 48 h and then washed twice with phosphate-buffered saline (PBS), fixed with 0.25% glutaraldehyde and stained with 0.1% 5-bromo-4 chloro-3 indolyl-beta-D-galactopyranoside (X-gal). Blue-stained cells were counted under a light microscope. Transduction efficiency was determined as the percentage of blue cells relative to viable cells.

2.6. Western blot analysis

Cells were harvested, washed twice with PBS, and lysed in lysis buffer (50 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% Nonident-P-40 (NP40), and completeTM (Boeringer Mannheim, GmbH, Germany)). Lysates were sonicated for 15 min and the supernatants were supplemented with gel loading buffer (0.13 mM Tris–HCl pH 6.5, 10% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue (BPB)). After boiling for 5 min, 30 μg aliquots of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels, transferred to polyvinylidene difluoride filters (Immobilon; Millipore, Tokyo, Japan) and blocked. Protein bands were probed with anti-p53 antibody (DO-7; DAKO, Glostrup, Denmark) diluted 1:1000 in PBS-Tween (PBST), anti-Bcl-2 antibody (clone 124; DAKO, Glostrup, Denmark) diluted 1:1000 in PBST, anti-BclXS/L antibody (S-18; Santa Cruz, CA, USA) diluted 1:1000 in PBST, anti-Bax antibody (N-20; Santa Cruz, CA, USA) diluted 1:1000, or anti-HA antibody (12CA5; Boeringer Mannheim, GmbH, Germany) diluted 1:80. Bands were visualised using an enhanced chemoluminescence method (ECL, Amersham Pharmacia Biotech, Tokyo, Japan).

2.7. Semi-quantitative RT-PCR analysis

Total cellular RNA was obtained from 1×10^6 cells by the standard guanidinium-thiocyanate-phenol-chloroform extraction method. One microgram of RNA was reverse-transcribed using a First-strand cDNA Kit (Amersham Pharmacia Biotech, Tokyo, Japan) in a total reaction volume of 15 μl. An aliquot (0.5 μl) of each cDNA product was used for PCR. PCR was performed according to the method previously described [20]. The nucleotide sequences of the primers used to amplify the *Bax*, *Bcl-2* and *Bcl-x* genes were as follows: *Bax*, sense primer 5′-TGA CGG CAA CTT AAC TGG G-3′ and antisense primer 5′-TTC CAG ATG GTG AGC GAG G-3′ [21], *Bcl-2*, sense primer 5′-ACA ACA TCG CCC TGT GGA TGA T- 3′ and antisense primer 5′-ATA GCT GAT TCG ACG TTT TGC C-3′, *Bcl-xL*, sense primer 5′-TTG GAC AAT GGA CTG GTT GA-3′ and antisense primer 5′-GTA GAG TGG ATG GTC AGT G-3′, respectively. The PCR condition consisted of denaturation for 5 min at 94°C, then 25 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 57°C and extension for 1 min at 72°C, followed by a final extension reaction for 5 min at 72°C. For semi-quantification of the mRNA level of the *Bax* gene, the PCR products were separated in 1% agarose gels containing an excess (2 ng/ml) of ethidium bromide, and the intensity of each band was measured using a FluorImager SI (Molecular Dynamics, Tokyo, Japan). The expression of *β2-microglobulin* was used as an internal control of the experiment [20]. To permit quantification, cDNA from a sample with high *Bax* expression was diluted 1:2, 1:4, 1:8, 1:16 and 1:32 in water and amplified, and the amounts of product were used to generate a standard curve. The relative quantity of *Bax* mRNA in the samples was calculated and normalised using this standard curve.

2.8. Cell morphology

At 48 h after transfection with AxCALNKYbax + AxCANcre, morphological changes were evaluated in comparison with non-treated cells by using standard phase-contrast microscopy. To visualise apoptosis, cells were fixed with 1% glutaraldehyde for 30 min, washed with PBS, and stained with Hoechst 33258 dye. The number of nuclear-fragmented cells was counted and the cells were photographed.

2.9. Cytotoxicity evaluation and quantitative assessment of apoptosis and Caspase-3 (CPP32) activity

Cells were plated in 96-well plates (1×10^3 cells/well). After 24 h, AxCALNKYbax + AxCANcre, or + Ax1w1 (control transfection) at 100 pfu/cell was transfected into the cell lines. After the cells were incubated for 1 day, 10 μM cisplatin or 0.1 μM paclitaxel (Bristol-Myers Squibb Company, Tokyo, Japan) was added, and the incubation was continued. The number of viable cells was then evaluated at various times using the WST-1 assay, as described above. Cells transfected

with AxCALNKYbax + AxCANcre were also incubated for 1 day, and a photometric enzyme-immunoassay of cytoplasmic histone-associated-DNA-fragments (Cell Death Detection ELISA, Boeringer Mannheim, GmbH, Germany) was performed to evaluate quantitatively the apoptosis. To evaluate Caspase-3 (CPP32) activity, 1×10^6 cells were pelleted after a 24-h incubation with AxCALNKYbax + AxCANcre, or + Ax1w1 (control transfection) at 100 pfu/cell, and 50 μg protein was assayed using a CPP32/Caspase-3 Colorimetric Protease Assay Kit (TaKaRa, Otsu, Japan).

2.10. Statistical analysis

For analysis of between-group differences, P values were determined using ANOVA. A P value less than 0.05 was considered significant.

3. Results

3.1. Mutational analysis

Each ovarian cancer cell line was assessed for *TP53* mutations by PCR/SSCP and direct sequencing. In SK-OV-3, neither mRNA nor protein expression of p53 was found (data not shown). This cell line has been reported to have a nonsense mutation and chromosome rearrangement [22] of the *p53* gene. OVCAR-3 possessed only a mutant allele in exon 7, compatible with previous findings of mutation at codon 248 (data not shown) [22]. No *p53* mutation was detected in A2780 (Fig. 1), which has been reported to have a wild-type *p53* gene [23]. A2780/cDDP, a cisplatin-resistant subline of A2780, contained a point mutation in exon 5 in one allele, while the other allele retained a wild-type *p53* gene (Fig. 1). Sequence analysis revealed a missense mutation from valine (GTT) to phenylalanine (TTT) at codon 172.

3.2. Sensitivity to cisplatin

The sensitivities of the four ovarian cancer cell lines to cisplatin treatment represented by IC 50 values were 22 μM cisplatin for SK-OV-3, 5 μM for OVCAR-3, 3 μM for A2780, and 25 μM for A2780/cDDP. Though OVCAR-3 has been reported as a cisplatin-resistant cell line, in our study the IC50 value was only 5 μM for OVCAR-3. Therefore, we regard OVCAR-3 as 'marginally cisplatin-resistant' in this study.

3.3. Endogenous expression of Bax, Bcl-2, Bcl-X_L and p53 following cisplatin treatment

The endogenous basal expression levels of Bax, Bcl-2, Bcl-X_L and p53 proteins in SK-OV-3, OVCAR-3, A2780 and A2780/cDDP were determined by Western blotting. The alterations in the expression levels of Bax, Bcl-2, Bcl-X_L and p53 after treatment with 10 μM cisplatin were also determined by semi-quantitative RT-PCR and Western blotting. As shown in Fig. 2, when the cells were cultured in standard medium to 80% confluence, the basal expressions of Bax were positive in A2780, A2780/cDDP, and OVCAR-3, and was weakly positive in SK-OV-3. The expressions of Bcl-2 and Bcl-X_L were strongly positive in A2780, A2780/cDDP and OVCAR-3 and weakly positive in SK-OV-3. As for p53 proteins, A2780, A2780/cDDP, and OVCAR-3 were positive, while SK-OV-3 was negative. When the cells were treated with cisplatin, Bax expression increased in A2780 and A2780/cDDP in a time-dependent manner, whereas no significant induction was observed in SK-OV-3 or OVCAR-3 (Figs. 2 and 3). The levels of induced Bax protein was intense in A2780, and less intensive in other cell lines. The expression of Bcl-2 and Bcl-X_L did not change significantly. Time-dependent increases in the expression of p53 occurred in A2780 and A2780/cDDP, while no significant induction was observed in OVCAR-3, and no expression was detected in SK-OV-3 (Fig. 2).

3.4. Transduction efficiency of adenoviral-mediated LacZ gene in ovarian cancer cell lines

In SK-OV-3, the transduction efficiency (the percentage of cells expressing beta-galactosidase) was 0% with no treatment, 4.8±2.4% at 1 pfu/cell, 23.7±2.9% at 10 pfu/cell, 92.7±4.0% at 50 pfu/cell, and 100% at 100 or 500 pfu/cell. Similarly, at 100 pfu/cell, the transduction efficiency was 100% in both OVCAR-3 and A2780/cDDP, and 81% in A2780. Therefore, subsequent

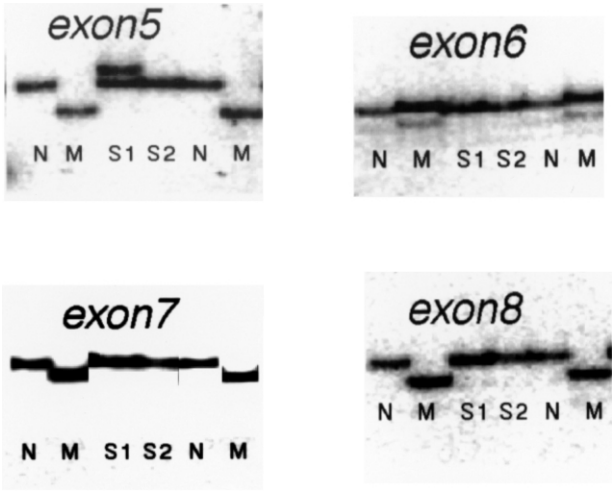

Fig. 1. The mutations in exons 5–8 of the *p53* gene were screened by polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP). N, normal (*p53* wild-type); M, mutant (*p53* mutation); S1, A2780/cDDP; S2, A2780.

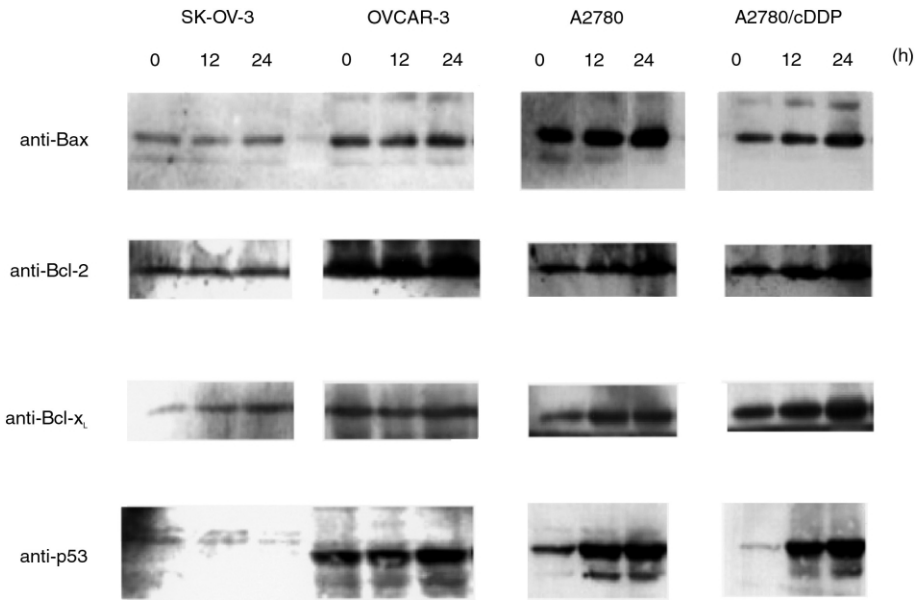

Fig. 2. Western blot analysis of endogenous expressions of Bax, Bcl-2 and Bcl-X_L following cisplatin treatment. Proteins were extracted after cells had been incubated 0, 12, or 24 h with cisplatin.

experiments were performed with viral infection at 100 pfu/cell.

3.5. *Bax expression following co-transfection of AxCALNKYbax-alpha and AxCANcre*

The co-transfection of AxCALNKYbax-alpha and AxCANcre induced expression of similar levels of Bax-α protein after a 72-h culture period in SK-OV-3, OVCAR-3, A2780 and A2780/cDDP. Because of the different levels of endogenous basal expression of Bax protein among the cell lines, the expression levels of Bax protein after cotransfection of AxCALNKYbax-alpha and AxCANcre ranged from approximately 2.4- to 19.4-fold higher than the endogenous expression levels in the respective cell lines (Fig. 4a). In the time course experiment in SK-OV-3 cells, Bax protein was scarcely detected before treatment, was clearly detected after a 12-h exposure to AxCALNKYbax-alpha + AxCANcre (Fig. 4b), and increased until it reached a plateau level at 24 h. The expression continued for at least 120 h after transfection (Fig. 4c). The exogenous expression was confirmed by immunoblot analysis using anti-HA tag antibody (Fig. 4b). No increase of Bax expression occurred when SK-OV-3 cells were treated with AxCALNKYbax + Ax1w1 (Fig. 4b, control transfection). Bcl-2 and Bcl-X_L expression were slightly induced after cotransfection of AxCALNKYbax-alpha and

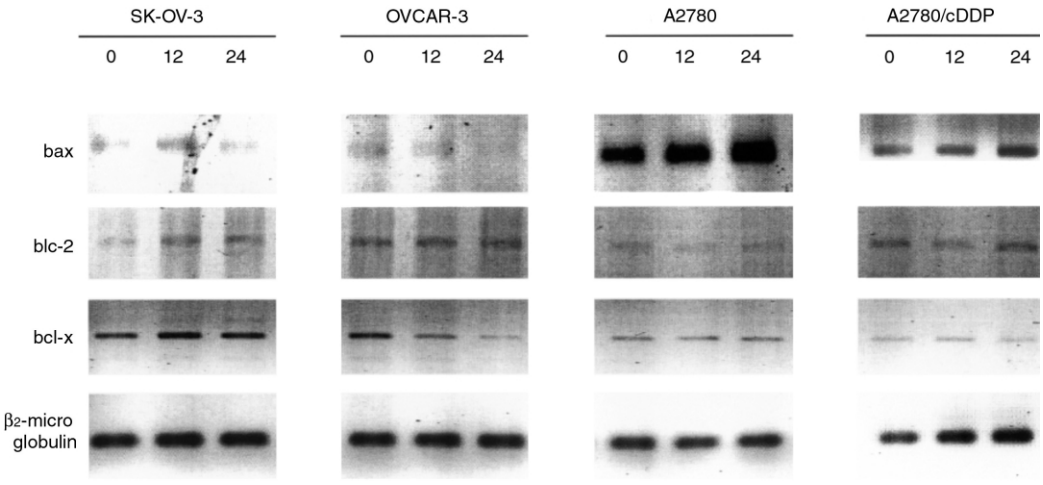

Fig. 3. Semi-quantitative RT-PCR showing endogenous mRNA levels of *Bax*, *Bcl-2* and *Bcl-X_L* following cisplatin treatment of SK-OV-3, OVCAR-3, A2780, and A2780/cDDP. RNA was extracted after cells had been incubated 0, 12, or 24 h with cisplatin.

AxCANcre in SK-OV-3, but no significant induction of Bcl-2 and Bcl-X_L expression was observed in OVCAR-3, A2780 and A2780/cDDP (Fig. 4a).

3.6. Changes in cell morphology

Morphological changes were observed after 48 h of treatment with AxCALNKYbax + AxCANcre. Apoptotic bodies and nuclear fragmentation were clearly found in OVCAR-3, A2780 and A2780/cDDP (Fig. 5a). However, there were no remarkable changes in the morphology of SK-OV-3. No morphological change was observed when cells were treated with AxCALNKYbax + Ax1w1 (control transfection). A significant increase in nuclear fragmentation was also observed using Hoechst 33258 dye-nuclear staining in ovarian cancer cells treated with AxCALNKYbax + AxCANcre (Fig. 5b). The proportion of apoptotic cells increased 12.9-fold after Bax induction in A2780 (control transfection versus *Bax* transfection were 5.7±0.4% versus 73.5±4.8%, $P < 0.05$), 5.8-fold in A2780/cDDP (14.7±5.0% versus 85.0±6.0%, $P < 0.05$), 17.5-fold in OVCAR-3 (4.0±0.4% versus 70.0±5.0%, $P < 0.05$), while it was only 1.7-fold in SK-OV-3 (3.0±0.4% versus 5.0±0.5%, non-significant) (Table 1).

3.7. Apoptotic effects of the Bax gene on ovarian cancer cells

The degree of apoptosis, evaluated by ELISA assay after treatment with AxCALNKYbax + AxCANcre, was high in the A2780 cells (6.07-fold increase compared with control transfection group, $P < 0.05$), A2780/cDDP (19.0-fold increase, $P < 0.05$), and OVCAR-3 (5.25-fold increase, $P < 0.05$). In contrast, SK-OV-3 showed a low degree of apoptosis at a MOI of 100 (1.61-fold increase, $P = 0.25$) (Fig. 6a, Table 1), but a marked apoptotic effect at MOI 1000 (15.2-fold increase, $P < 0.05$) (data not shown). Elevation of Caspase-3 (CPP32) activity also indicated the induction of apoptosis in A2780, A2780/cDDP and OVCAR-3; however, the induction rate of Caspase-3 activity was lowest in SK-OV-3 (MOI 100) (Fig. 6b). SK-OV-3 showed 3.46-fold elevation of Caspase-3 (CPP32) activity only when exposed to a higher MOI of 1000 (data not shown).

3.8. Cytotoxic effect of ovarian cancer cells with exogenous Bax expression

The cytotoxic effects of the transfected *Bax* gene after 48 h of incubation on A2780, A2780/cDDP, OVCAR-3, and SK-OV-3 were evaluated using a WST-1 assay. As shown in Fig. 7, A2780, A2780/cDDP, and OVCAR-3 showed dose-dependent suppression of the viable cell number after exposure to AxCALNKYbax + AxCANcre: a 40% reduction of cell number was obtained for A2780 compared with the control transfection group, and 50% for A2780/cDDP, 64.8% for OVCAR-3 (MOI 100) after 48 h of incubation with the Bax gene. The reduction rate of *Bax* transfection group was calculated as (1−Bax transfection group = control transfection group)×100. In contrast, only a 12.3% reduction of the cell number

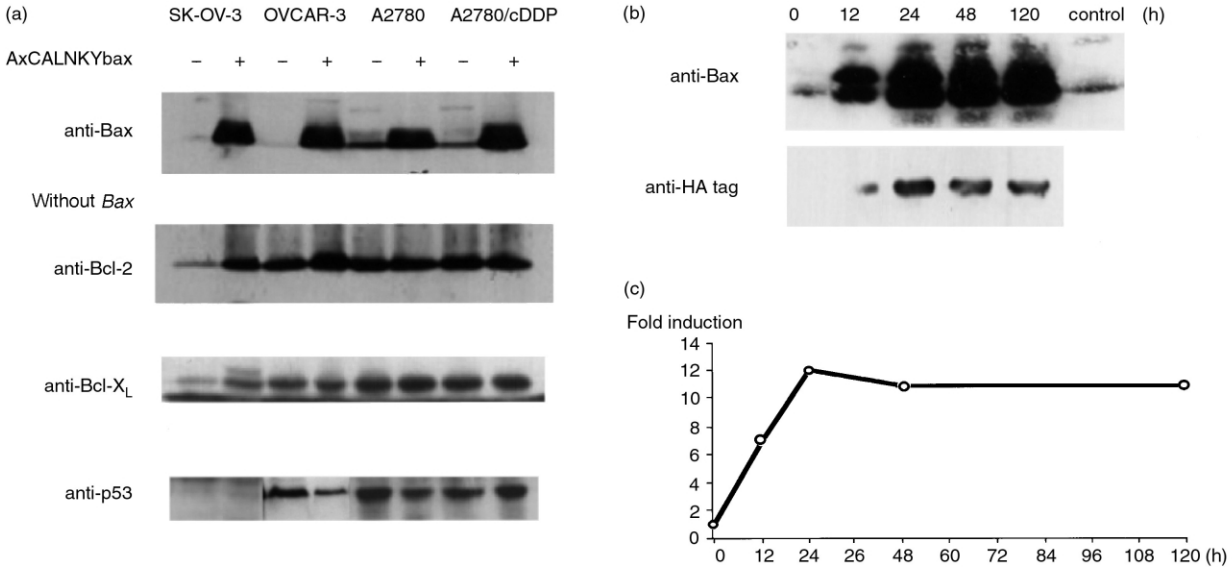

Fig. 4. Western blot analysis: (a) Bax expression in SK-OV-3, OVCAR-3, A2780, and A2780/cDDP following cotransfection of AxCALNKYbax-alpha and AxCANcre. (b) Time course of Bax expression (0–120 h) following cotransfection of AxCALNKYbax-alpha and AxCANcre probed with anti-Bax antibody in SK-OV-3 (upper lanes), and time course (0–120 h) of exogenous Bax expression probed with anti-HAtag antibody (lower lanes). Control, transfection of control virus (AxCALNKYbax + Ax1w1). (c) Quantitative assessment of Western blot analysis using NIH Image software: time course (0–120 h) of Bax expression following cotransfection of AxCALNKYbax-alpha and AxCANcre probed with anti-Bax antibody in SK-OV-3.

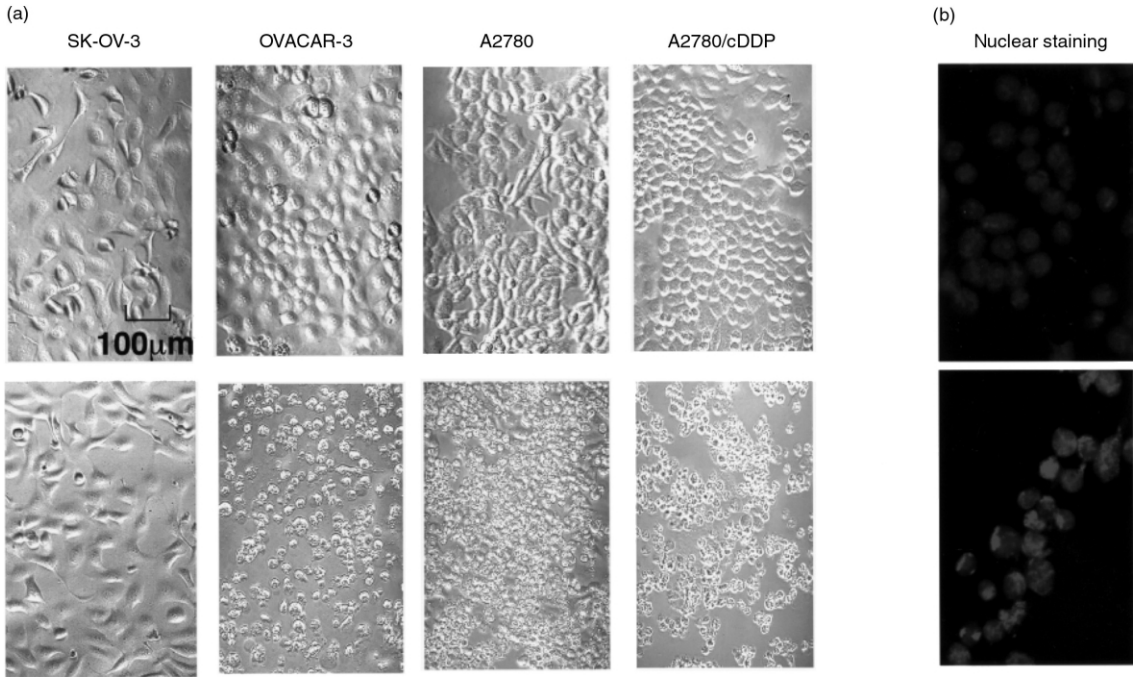

Fig. 5. (a) Morphological findings for SK-OV-3, OVCAR-3, A2780 and A2780/cDDP which had been transfected with (lower panel) or without (upper panel) the *Bax* gene; (b) nuclear staining with Hoechst 33258 dye in A2780 which was transfected with (lower) or without (upper) the *Bax* gene.

was obtained for SK-OV-3 cells (MOI 100) (Table 1). A 34.8% reduction of the cell number was obtained for SK-OV-3 only when exposed to the higher MOI of 1000 (Fig. 7).

3.9. Increased cytotoxicity of Bax introduction combined with chemotherapy

Treatment with cisplatin or paclitaxel combined with Bax introduction caused different cytotoxic effects depending on the cell line (Fig. 8 and Table 2). In the cisplatin-sensitive cell line A2780, reduction in the viable cell number was significantly higher in the cisplatin + Bax group compared with cisplatin-treatment alone or cisplatin + control transfection group ($P < 0.05$), and was also significantly higher in the paclitaxel + Bax group compared with paclitaxel alone or paclitaxel + control transfection group ($P < 0.05$) (Table 2 and Fig. 8). Similarly, in cisplatin-resistant A2780/cDDP and in marginally cisplatin-resistant OVCAR-3, reduction in the viable cell number was highest when it was treated in combination of Bax + -cisplatin or paclitaxel (Table 2 and Fig. 8). In cisplatin-resistant SK-OV-3, reduction in the viable cell number was slightly higher in combination of Bax + cisplatin or paclitaxel compared with the control groups.

Table 1

	% of cell survival		Apoptotic cells		% of apoptotic cells		
Cell line	Control	Bax	Control	Bax	No treatment	Control	Bax
A2780	100.0±8.8	60.0±0.0[a]	1.5±0.4	9.1±0.3[a]	1.5±0.4	5.7±0.4	73.5 ±4.8[b]
A2780/cDDP	100.0±1.8	50.0±1.8[a]	0.8±0.1	13.0±1.6[a]	6.4±6.7	14.7±5.0	85.0±6.0[b]
OVCAR-3	98.1±2.6	34.5±6.7[a]	0.9±0.2	4.6±0.4[a]	5.0±0.4	4.0±0.4	70.0±5.0[b]
SK-OV-3	95.8±3.3	84.0±7.8	0.7±0.0	1.2±0.0	4.0±0.4	3.0±0.4	5.0±0.5

Percentage of cell survival was evaluated by WST-1 assay. A2780, A2780/cDDP, OVCAR-3, and SK-OV-3 were treated with AxCALNKYbax-alpha at a multiplicity of infection (MOI) of 100. Absorbance (A490 nm/A650 nm) by WST-1 assay in no treatment group was calculated as 100%. Absorbance (A490 nm/A650 nm) by WST-1 assay in the other groups were calculated as a percentage against the no treatment group (see Fig. 7). Apoptotic cells following Bax introduction were evaluated by enzyme-linked immunosorbent assay (ELISA) assay in A2780, A2780/cDDP, OVCAR-3 and SK-OV-3. The results are expressed relative to the no treatment group (see Fig. 6a). Percentage of apoptotic cells from whole cells was determined by counting of the nuclear-fragmented cells stained with Hoechst 33258 dye. Control, transfection of control virus (AxCALNKY-bax + Ax1w1); Bax, transfection of AxCALNKYbax + AxCANcre. The values are expressed as mean ± standard deviation (S.D.)

[a] $P < 0.05$ against control.

[b] $P < 0.05$ against both no treatment and control.

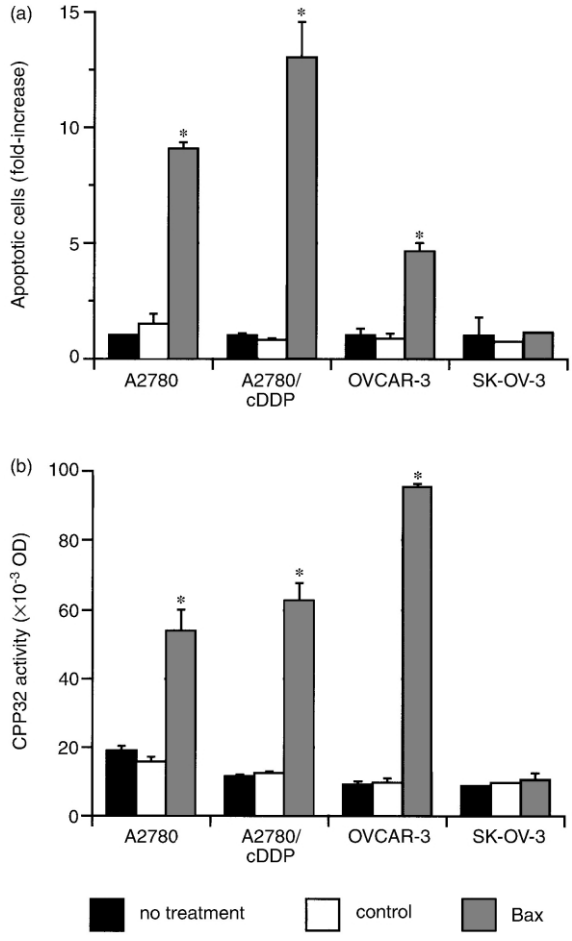

Fig. 6. (a) Apoptotic effects of *Bax* transfection evaluated by ELISA assay in A2780, A2780/cDDP, OVCAR-3, and SK-OV-3. control, transfection of control virus (AxCALNKYbax + Ax1w1); Bax, transfection of AxCALNKYbax + AxCANcre. The results are expressed relative to untreated control; bars, S.D. *, $P < 0.05$ for Bax versus control. (b) Caspase-3 (CPP32) activity following cotransfection of AxCALNKYbax-alpha and AxCANcre into A2780, A2780/cDDP, OVCAR-3, and SK-OV-3. control, transfection of control virus (AxCALNKYbax + Ax1w1); Bax, transfection of AxCALNKYbax + AxCANcre; bars, S.D. *, $P < 0.05$ for Bax versus control.

4. Discussion

Adenovirus-mediated gene transfer is known to be a powerful tool for *in situ* gene induction both in cancer cell lines *in vitro* and in cancer tissues *in vivo* [14]. In this study, we constructed a recombinant adenovirus which highly expressed the *Bax* gene. The intrinsic level of expression of Bax protein varied among the ovarian cancer cell lines examined. Regardless of the endogenous Bax levels, adenovirus-mediated transfer of the *Bax* gene induced a uniformly high level of Bax protein in these ovarian cancer cells, ranging from 2.4- to 19.4-fold higher than the respective endogenous levels. These levels were comparable to those obtained by stable transfection [4]. *Bax* gene transfer induced apoptosis efficiently in the cisplatin-sensitive cell line A2780, the marginally resistant OVCAR-3 and also in the cisplatin-resistant cell line A2780/cDDP. In contrast, a pro-apoptotic effect was minimal in SK-OV-3. Bax induction also led to marked activation of Caspase-3 (CPP32) in A2780, A2780/cDDP and OVCAR-3, but the activation was not prominent in SK-OV-3. Similarly, the cytotoxic effect of *Bax* indicated as the reduction in cell number was 40, 50 and 64.8% in A2780, A2780/cDDP and OVCAR-3, respectively, while it was only 12.3% in the SK-OV-3 cells at a viral MOI of 100. This is consistent with a recent report on the effect of adenoviral *Bax* transfer in SK-OV-3 cells, resulting in less than 5% cytotoxicity for MOI of 100 and approximately 50% cytotoxicity for MOI of 500 [15]. Exogenous Bax expression did not alter significantly the levels of Bcl-2 and Bcl-X_L in OVCAR-3, A2780, and A2780/cDDP, except for a slight induction in SK-OV-3. Thus, the therapeutic effect of adenovirus-mediated transfer of the *Bax* gene appeared to be sufficient in some, but not all, types of ovarian cancer.

To clarify further why these cell lines differ in the cytotoxicity resulting from *Bax* transfection and in cisplatin sensitivity, we evaluated the intracellular apoptotic signal induced by cisplatin. Cisplatin increased the *Bax* level in cisplatin-sensitive A2780. Compared with this, the induced level of *Bax* after cisplatin treatment was weak in the more cisplatin-resistant cell lines, OVCAR-3, A2780/cDDP or SK-OV-3. These findings may indicate that cisplatin resistance is, to some extent, correlated with attenuated induction of Bax protein following cisplatin treatment. The expression of Bcl-2 and Bcl-X_L did not significantly change following cisplatin treatment in all the cell lines. The mutation of the tumour suppressor *p53* gene is often associated with cisplatin-resistance. SSCP/sequencing analysis in this study revealed that A2780 has the wild-type *p53* gene, which was mutated in A2780/cDDP and OVCAR-3, and entirely deleted in SK-OV-3. Accumulation of p53 protein was detected in A2780 after cisplatin treatment. It is well known that the DNA damage elicited by cisplatin leads to accumulation of intracellular p53 protein, which transcriptionally activates specific genes such as *p21* and *Bax* [24]. In this process, induction of Bax is considered to play a major role in the apoptotic process related to cisplatin treatment [9,25]. Thus the cisplatin-sensitive A2780 cell line in this study probably retains the apoptosis pathway mediated by *p53* and *Bax*. However, this pathway may have been impaired in the cisplatin-resistant cell lines used here, which would have resulted in cisplatin resistance, with cancer cells avoiding apoptosis. Therefore, supplemental Bax expression may restore apoptosis in these cell lines.

This is the first report to show the combination effect of adenovirus-mediated induction of Bax protein with conventional chemotherapeutic reagent such as cisplatin

Table 2

Cell line	% of cell survival						
	No treatment	CDDP	Control + CDDP	Bax + CDDP	Paclitaxel	Control + paclitaxel	Bax + paclitaxel
A2780	100.0±5.6	61.0±10.2	72.4±15.0	13.0±6.5[a]	79.5±8.6	62.7±0.1	30.4±4.5[c]
A2780/cDDP	100.0±12.9	100.0±10.9	75.3±0.5	38.0±4.6[a]	62.6±9.1	41.0±0	20.1±1.7[c]
OVCAR-3	100.0±3.4	43.0±4.3	52.5±5.0	9.0±0.8[a]	80.3±4.1	70.0±2.0	12.2±0.1[c]
SK-OV-3	100.0±2.5	100.0±3.3	89.1±1.8	83.0±3.4[b]	82.6±0.5	80.0±1.8	67.8±1.3[c]

[z] Percentage of cell survival was evaluated by WST-1 assay after bax induction with or without cisplatin and paclitaxel treatment. A2780, A2780/cDDP, OVCAR-3 and SK-OV-3 cells were treated with AxCALNKYbax-alpha at a multiplicity of infection (MOI) of 100. Absorbance (A490 nm/A650 nm) by WST-1 assay in the no treatment group was calculated as 100%. Absorbance (A490 nm/A650 nm) by WST-1 assay in the other groups were calculated as a percentage against the no treatment group (see Fig. 8). CDDP, cisplatin treatment; control + CDDP, transfection of AxCALNKYbax + Ax1w1 with cisplatin treatment; Bax + CDDP, transfection of AxCALNKYbax + AxCANcre with cisplatin treatment; paclitaxel, paclitaxel treatment; control + paclitaxel, transfection of AxCALNKYbax + Ax1w1 with paclitaxel treatment; Bax + paclitaxel, transfection of AxCALNKYbax + AxCANcre with paclitaxel treatment. The values are expressed as means ± standard deviation (S.D.).

[a] $P < 0.05$ against both CDDP and control + CDDP.

[b] $P < 0.05$ against CDDP.

[c] $P < 0.05$ against both paclitaxel and control + paclitaxel.

or paclitaxel. In combination with paclitaxel, the pro-apoptotic effect of *Bax* transfer was enhanced even in the cisplatin-resistant cell line A2780/cDDP. However, in another cisplatin-resistant cell line, SK-OV-3, *Bax* transfer did not induce sufficient cytotoxicity effects, whereas a mild additive effect was obtained by combining of *Bax* with cisplatin or paclitaxel. Therefore, *Bax* gene transfer would be a more useful tool when it is

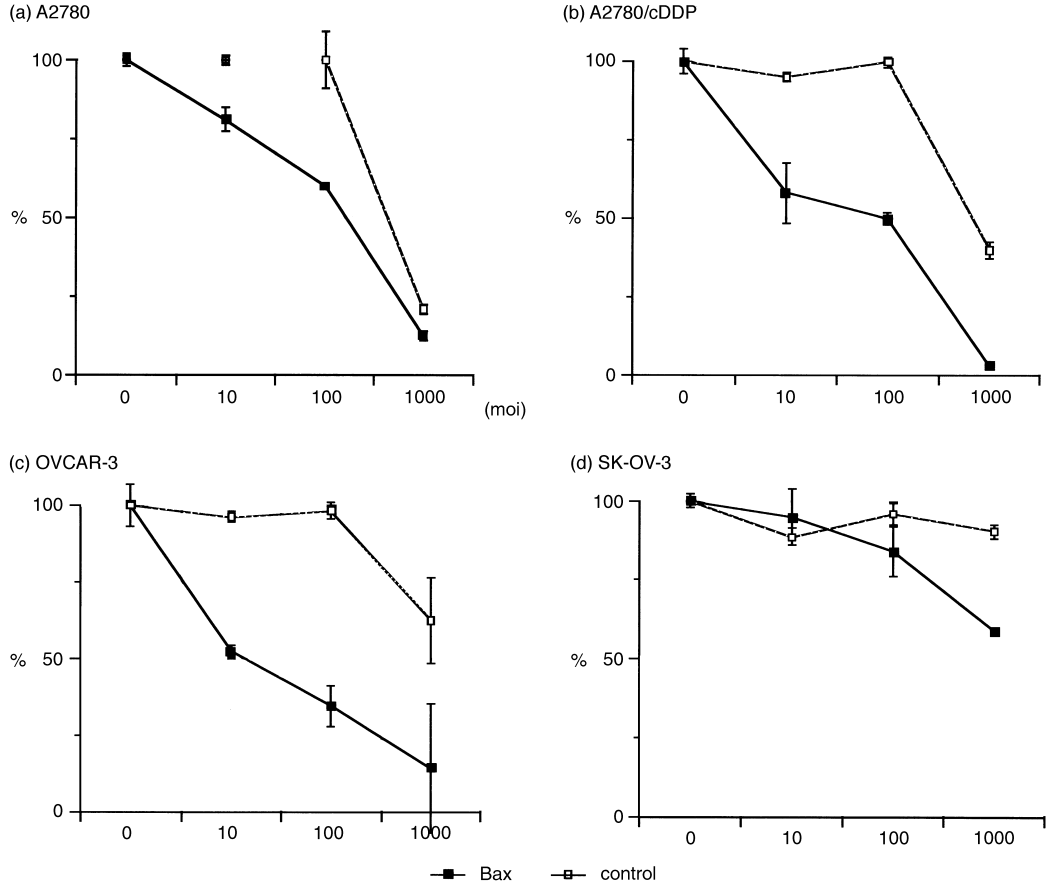

Fig. 7. WST-1 assay to evaluate the cytotoxic effects of exogenous *Bax* gene on (a) A2780, (b) A2780/cDDP, (c) OVCAR-3 and (d) SK-OV-3. A2780, A2780/cDDP, OVCAR-3, and SK-OV-3 were treated with AxCALNKYbax-alpha ranging from MOI 10 to MOI 1000. Absorbance (A490 nm/ A650 nm) by WST-1 assay in no treatment group was calculated as 100%. Absorbance (A490 nm/A650 nm) by WST-1 assay in the other groups were calculated in percentage against no treatment group; bars, S.D. Bax, transfection of AxCALNKYbax + AxCANcre; control, transfection of control virus (AxCALNKYbax + Ax1w1).

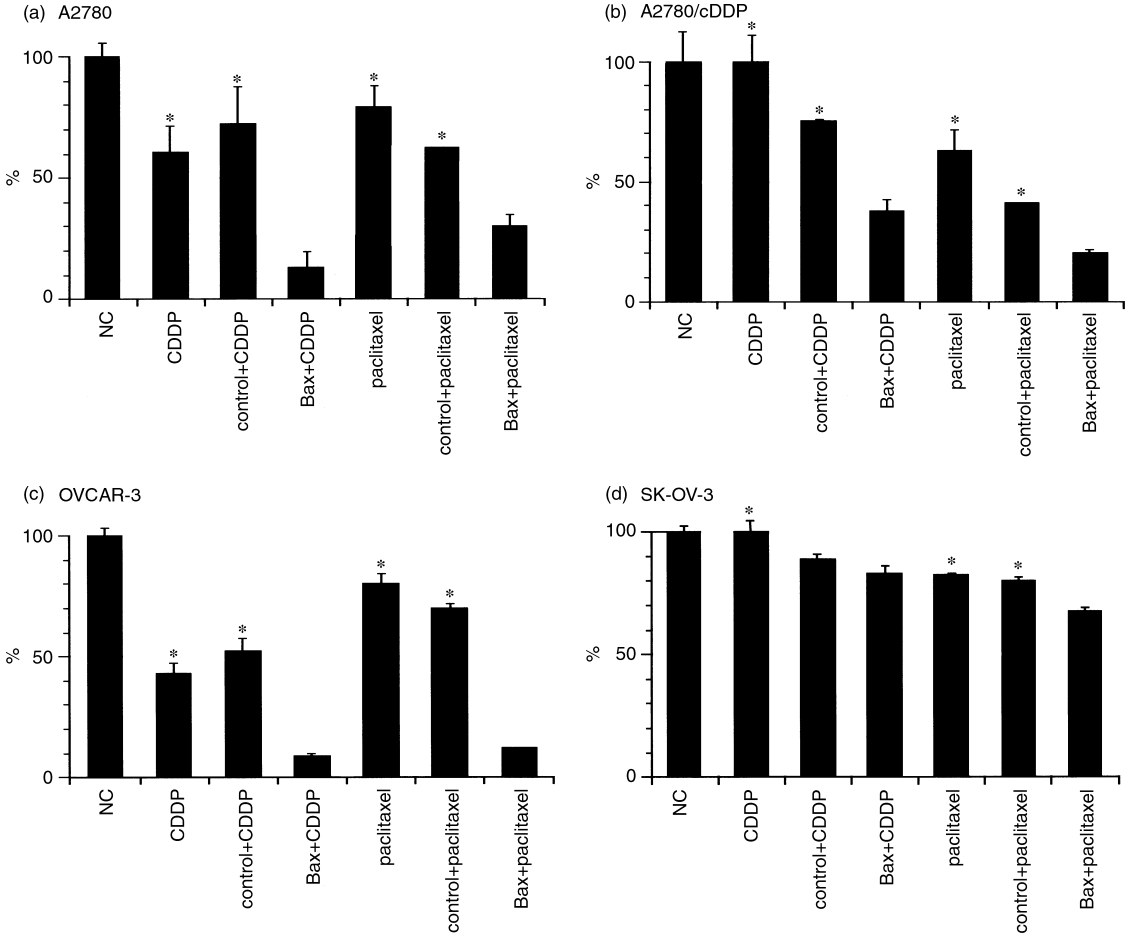

Fig. 8. Cytotoxic effects were evaluated by WST-1 assay after Bax induction with or without cisplatin and paclitaxel treatment of A2780, A2780/cDDP, OVCAR-3, and SK-OV-3. A2780, A2780/cDDP, OVCAR-3, and SK-OV-3 were treated with AxCALNKYbax-alpha at a MOI of 100. Absorbance (A490 nm/A650 nm) by WST-1 assay in the no treatment group was calculated as 100%. Absorbance (A490 nm/A650 nm) by the WST-1 assay in the other groups were calculated as a percentage against the no treatment group; bars, S.D. NC, no treatment; CDDP, cisplatin treatment; control + CDDP, transfection of AxCALNKYbax + Ax1w1 with cisplatin treatment; Bax + CDDP, transfection of AxCALNKYbax + AxCANcre with cisplatin treatment; control + paclitaxel, transfection of AxCALNKYbax + Ax1w1 with paclitaxel treatment; Bax + paclitaxel, transfection of AxCALNKYbax + AxCANcre with paclitaxel treatment. (a, b, c) $*P < 0.05$ for: CDDP versus Bax + CDDP; control + CDDP versus Bax + CDDP; paclitaxel versus Bax + paclitaxel; control + paclitaxel versus Bax + paclitaxel; (d) $*P < 0.05$ for: CDDP versus Bax + CDDP; paclitaxel versus Bax + paclitaxel; control + paclitaxel versus Bax + paclitaxel.

combined with cisplatin or paclitaxel in chemoresistant ovarian cancer.

In conclusion, this study revealed that transfer of the pro-apoptotic *Bax* gene effectively caused apoptosis and cytotoxicity in chemoresistant ovarian cancer cell lines. Thus adenoviral-mediated transfer of the *Bax* gene may be promising gene therapy in chemoresistant ovarian cancer for use alone or in combination with anticancer drugs.

Acknowledgements

We thank Dr I. Saito (Institute of Medical Science, University of Tokyo) for the generous gifts of pAxCALNLw cosmid vector, Ax1w1, and AxCALacZ adenovirus. This work was supported by Grants-in-Aid for Scientific Research to I.K. (10877257, 09470358) and M.M. (09771274) from the Ministry of Education, Science, Culture and Sports, Japan.

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