The potent peptide antagonist to angiogenesis, C16Y, and cisplatin act synergistically in the down-regulation of the Bcl-2/Bax ratio and the induction of apoptosis in human ovarian cancer cells

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Abstract. Cisplatin is one of the most potent antitumor agents for ovarian cancer, but has also been implicated in normal tissue cytotoxicity. We examined the effect of cisplatin alone and in combination with C16Y, a newly-identified anti-angiogenic peptide from the NH_2-terminal domains of the γ-chain of laminin-1, on the modulation of Bcl-2/Bax expression and induction of apoptosis in ovarian cancer cells (OVACAR3). C16Y did not elicit cell death of human umbilical vein endothelial cells (HUVECs). Cisplatin exerted a lethal effect with an EC_{50} of 10 μM in OVACAR3s. In the presence of 25 or 50 μg/ml of C16Y (a range which has no effect against HUVECs), the EC_{50} for cisplatin in OVACAR3s decreased to 3.5 and 2.0 μM, respectively. Using fluorescence-activated cell sorting (FACS) analysis of DNA stained OVACAR3s and terminal deoxynucleotide tranferase-mediated dUTP nick end-labeling (TUNEL), we found that even at concentrations of 1 and 3 μM cisplatin, C16Y at 10 and 25 μg/ml increased the incidence of apoptosis in OVACAR3s by 3-5-fold. Each drug had some measurable effect on Bax protein expression. Furthermore, Bcl-2 protein expression levels were markedly reduced by C16Y alone and cisplatin alone in a dose-dependent manner. The combination of C16Y and cisplatin resulted in a further dramatic reduction in Bcl-2, underscoring the pronounced synergy produced by cisplatin and C16Y together. On the other hand, C16Y did not activate any other signal transduction pathways that usually culminate in the activation of apoptosis, such as the p53, p21^{waf1}, p73, ERK1/2 or PI3-AKT pathways. These observations suggest that the suppression of the Bcl-2/Bax ratio may play an important role in mediating the synergistic effect of cisplatin and C16Y on the induction of apoptosis in OVACAR3 cells.

Introduction

Cisplatin is the accepted treatment of choice for ovarian cancer, and acts by inhibiting DNA synthesis, affecting the cell cycle, and inducing apoptosis. Unfortunately, the high concentrations used in chemotherapy are highly toxic to normal cells and tissue. Therefore, in an effort to reduce the dose and cytotoxicity of cisplatin in normal cells, the use of cisplatin in combination with other drugs has been examined in experimental models (1).

The basement membrane-derived glycoprotein, laminin-1, is very biologically active. It increases cell adhesion, tumor growth, and angiogenesis via multiple active sites (2,3). Many sites for angiogenesis have been identified on laminin-1, but only one site, YIGSR, has been found to be an inhibitor of angiogenesis, and this site is located on the β1 chain (4). Recently, C16Y, which is a 12-mer peptide from the NH_2-terminal domain of the γ-chain of laminin-1, has also been found to have an anti-angiogenic effect (5).

As knowledge on the multistep process of angiogenesis accumulates, more is being understood about how other pathways within the cell that have to be dysregulated in cancer, such as apoptosis, interact with angiogenesis (6). We hypothesized that C16Y would have a synergistic effect with cisplatin on cell death in ovarian cancer via induction of apoptosis and thus we examined the effect of C16Y against human ovarian cancer cells.

Materials and methods

Peptides. C16Y and C16C (control peptide) were synthesized by the CBER Facility for Biotechnology Resources (Food and Drug Administration, Bethesda, MD) as previously described (5).

Cell culture. The human ovarian cancer cell line (OVACAR3), human umbilical endothelial cells (HUVECs) and the mouse fibroblast cell line (NIH/3T3) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in complete DMEM/F12 medium supplemented with 5% FBS,

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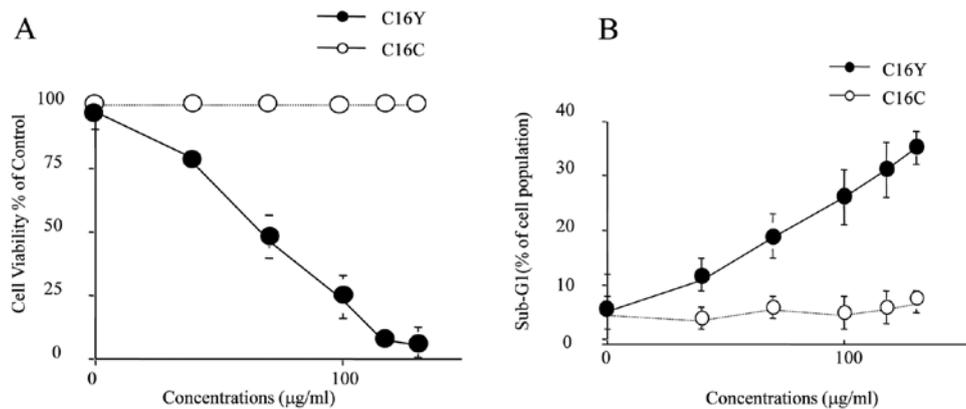E-mail: yyoshida@u-fukui.ac.jp

Figure 1. Inhibition of cell growth and induction of apoptosis in OVACAR3 cells by C16Y. (A) The MTT assay was performed after 24 h exposure with various concentrations of C16Y without FCS. Each point represents the mean ± SD of three replicates. (B) OVACAR3s were incubated for 24 h at 37°C in DMEM/F12 medium with various concentrations of C16Y without FCS. Cells were fixed with methanol, stained with propidium iodide, and examined by FACS. Data represent the mean ± SD for duplicate assays in triplicate plates. Significant differences were observed after treatment with C16Y starting at a concentration of 50 μg/ml in both (A) and (B) (P<0.001).

penicillin and streptomycin (Invitrogen, Carlsbad, CA). The cell lines were routinely maintained at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies. The primary mouse monoclonal antibodies anti-p21^{waf1}, anti-p53, anti-Bax and anti-Bcl-2 were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Phospho-ERK1/2, phospho-PI3K and phospho-AKT(T308) were purchased from Cell Signaling (Danvers, MA). Monoclonal mouse anti-actin was purchased from Sigma Israel Chemical, Ltd. (Danvers, MA). Both goat anti-mouse and goat anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase (HRP) were obtained from Biomakor (Danvers, MA).

Reagents. Cisplatin (cis-diamminedichloroplatinum II) was a kind gift from Nippon Kayaku Co., Ltd. (Tokyo, Japan). MTT (3-(4,5-dimethylthiazol-2,5-diphenyltetorazolium bromide) was purchased from Sigma Chemical Co. (St. Louis, MO).

MTT assay for cytotoxicity. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetorazolium bromide) colorimetric assay was used to quantify the chemosensitivity of the cells to cisplatin, C16Y and C16C and to the combination of C16Y and cisplatin. In brief, HUVECs and OVACAR3s were plated in all the wells of a 96-well plate at a density of 4,000 cells per well, and incubated overnight to allow attachment. The next day the cells were treated for 24 or 48 h with various concentrations of cisplatin (1-20 μM), C16Y (0-200 μg/ml), C16C (from 0-200 μg/ml) or with a combination of C16Y and cisplatin. Cells were incubated with each concentration (10, 25 and 50 μg/ml) of C16Y alone or combined with increasing concentrations of cisplatin (1, 5, 10 and 20 μM) after which 20 μM of 5 mg/ml MTT in PBS was added to each well and the plate was incubated for a further 4 h at 37°C. In this method, MTT is reduced to a crystalline blue-colored product (formazan) by the intracellular dehydrogenase of viable cells. This formazan is dissolved in dimethyl sulfoxide (DMSO), and can then be measured spectrophotometrically. Absorbance, measured as optical density (OD), at 540 nm was recorded. The cell survival

percentages were calculated by dividing the mean OD of drug-treated cells by that of the control cells. The IC$_{50}$ (50% inhibitory concentration) values were the drug concentrations that induced a 50% reduction in control cells (7).

Fluorescence-activated cell sorting (FACS) analysis. Floating cells and trypsinized attached cells were collected after each drug treatment and combined in order to ensure a complete representation of the cell population (8). Cells were washed with cold PBS and fixed in cold methanol (-20°C) for 1 h. Subsequently, cells were centrifuged, resuspended in 0.5 ml cold PBS and stained for at least 15 min with 50 μg/ml propidium iodide in the presence of 100 μg/ml RNase A. Cells were then analyzed in the fluorescence-activated cell sorter (FACSort; Becton-Dickinson). For the analysis, 5,000 events from the gated sub-population were recorded separately.

Western blot analysis. Cells were washed with cold PBS and harvested with rubber policemen using lysis buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10 μg/ml leupeptin, 10% glycerol, 30 mM NaF, 30 mM sodium pyrophosphate, 1 mM orthovanadate and 5 μg/ml aprotinin. Lysates were then boiled in sample buffer for 10 min and samples containing equal amounts of protein (30-200 μg) were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The blots were then blocked using 5% milk powder in PBS plus 0.05% Tween-20 and reacted overnight at 4°C with the corresponding primary antibodies, followed by a 1-h incubation at room temperature with goat anti-rabbit IgG conjugated to HRP. Detection was performed using the enhanced chemiluminescence (ECL) kit (Amersham Co., Buckinghamshire, UK).

Phase contrast and immunofluorescent microscopy. Phase contrast and fluorescent microscopy was performed on cells with DNA stained by the terminal deoxynucleotide transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method, which reveals fragmented DNA *in situ* (9). Cultures were fixed
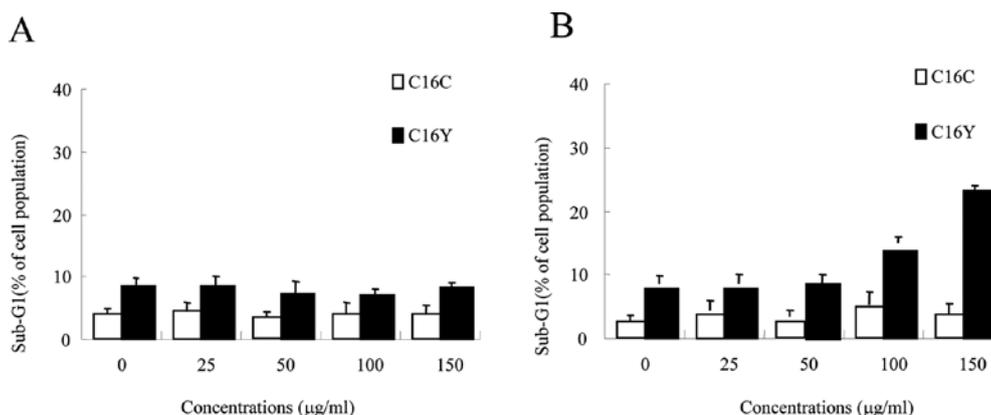
Figure 2. Cell growth inhibition in human umbilical vein endothelial cells (HUVECs) and a mouse fibroblast cell line (NIH/3T3). (A) HUVEC and NIH/3T3 cells were incubated for 24 h at 37˚C in DMEM/F12 medium in the presence of increasing concentrations of C16Y. Cells were fixed with methanol, stained with propidium iodide, and examined by FACS. Data represents mean ± SD for duplicate assays in triplicate plates.
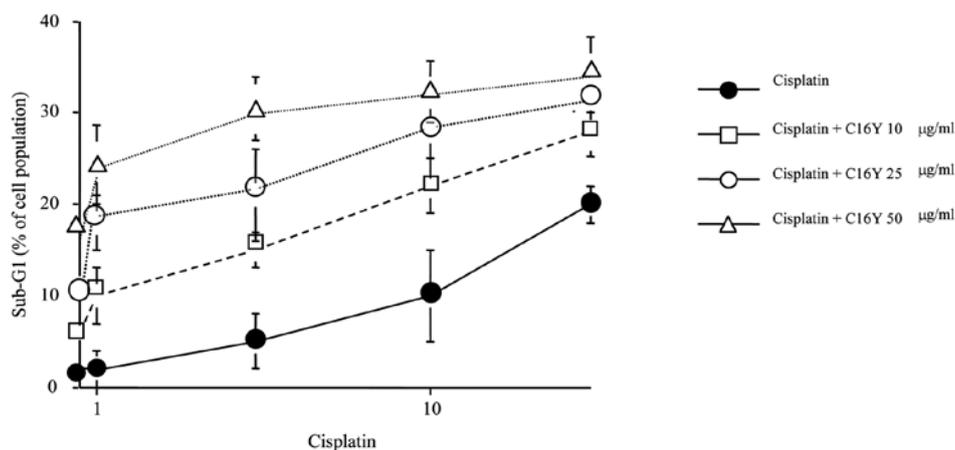

Figure 3. Synergistic induction of apoptosis by C16Y and cisplatin in OVACAR3 cells. Cells were incubated with each concentration (10, 25 and 50 µg/ml) of C16Y alone or combined with increasing concentrations of cisplatin (from 1-20 µM) for 24 h in DMEM/F12 medium without FCS. Cells were fixed with methanol, stained with propidium iodide and examined by FACS. The data are expressed as the sub-G1 population, which serves as an index for the incidence of apoptosis. Values of cisplatin-C16Y (10 and 25 µg/ml) treated cells are significantly different from cells treated with cisplatin alone at 1-10 µM (P<0.001).

with 3% paraformaldehyde in PBS (pH 7.4) and permeabilized for 2 min with 0.1% Triton X-100 in 0.1% sodium citrate at 0˚C. TUNEL staining was performed using the *In Situ* Cell Death Detection kit (Boehringer, Mannheim, Germany). Microscopic examination of the specimens was performed using a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) in both phase and fluorescent modes.

Statistical analysis. Analysis of the percentage of cell viability and densitometer tracing was performed using the t-test for comparison of means. Differences between treatment groups were considered statistically significant at P<0.05.

Results

First we examined the sensitivity of OVACAR3 ovarian cancer cells to C16Y, which is a 12-mer peptide from the NH_2-terminal domain of the γ-chain of laminin-1. In order to establish the effect of C16Y on the viability of OVACA3s, increasing concentrations of C16Y were introduced into the cell cultures for 24 h.

The IC_{50} of C16Y for OVACAR3s was 50 µg/ml (Fig. 1A). While only a small induction in apoptosis was observed at 25 µg/ml of C16Y, a pronounced induction of apoptosis was evident at 50 µg/ml of C16Y; a dramatic increase with increasing doses of the drug (Fig. 1B) was observed, which could explain the loss of cell viability at high concentrations of the drug.

The next step was to determine whether C16Y affects normal cells, namely HUVECs and a mouse fibroblast cell line (NIH/3T3) in a dose-dependent manner. C16Y did not elicit HUVEC or NIH/3T3 cell apoptosis at concentrations up to 50 µg/ml (Fig. 2).

We then examined whether co-stimulation of OVACAR3 cells with the combination of cisplatin and C16Y can exert a synergistic effect of apoptosis at relatively low concentrations, which may not be toxic to normal cells and tissues. Cisplatin had an EC_{50} of 10 µM against the OVACAR3s and was lethal at this concentration. Fig. 3 shows the induction of apoptosis in OVACAR3 cells by C16Y and cisplatin on the dose response curve. The dose-response curve obtained in the presence of increasing concentrations of C16Y appears to be shifted to the
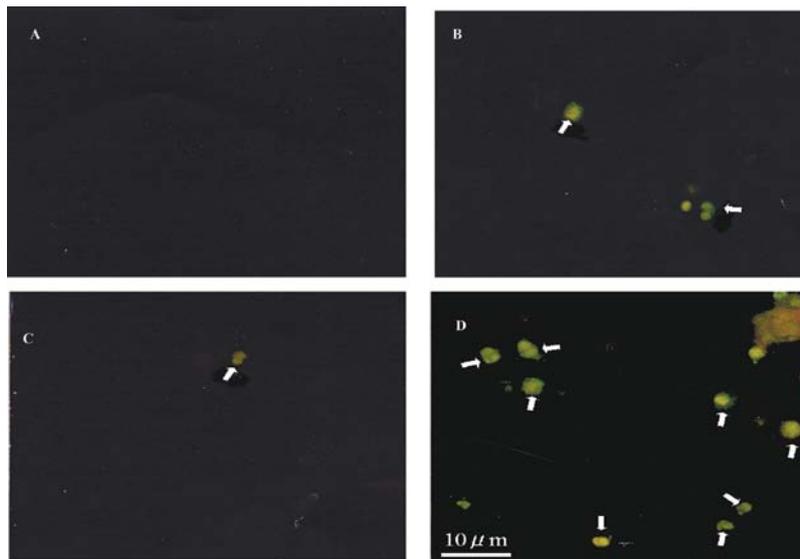
Figure 4. Apoptosis in OVACAR3 cells incubated with C16Y and/or cisplatin. Cells were incubated at 37°C (A) without FCS for 24 h or (B) in the presence of 25 μg/ml C16Y alone, or (C) 1 μM cisplatin alone, or (D) with a combination of C16Y and cisplatin at the above concentrations. Cells were fixed with 3% paraformaldehyde and stained for apoptotic nuclei according to the TUNEL method. (A-D) Fluorescent microscopy of identical fields. Note the low incidence of apoptotic nuclei (green fluorescence, wide arrows) following treatment with C16Y alone or cisplatin alone and the high incidence of apoptotic nuclei in cell cultures co-stimulated with C16Y and cisplatin.
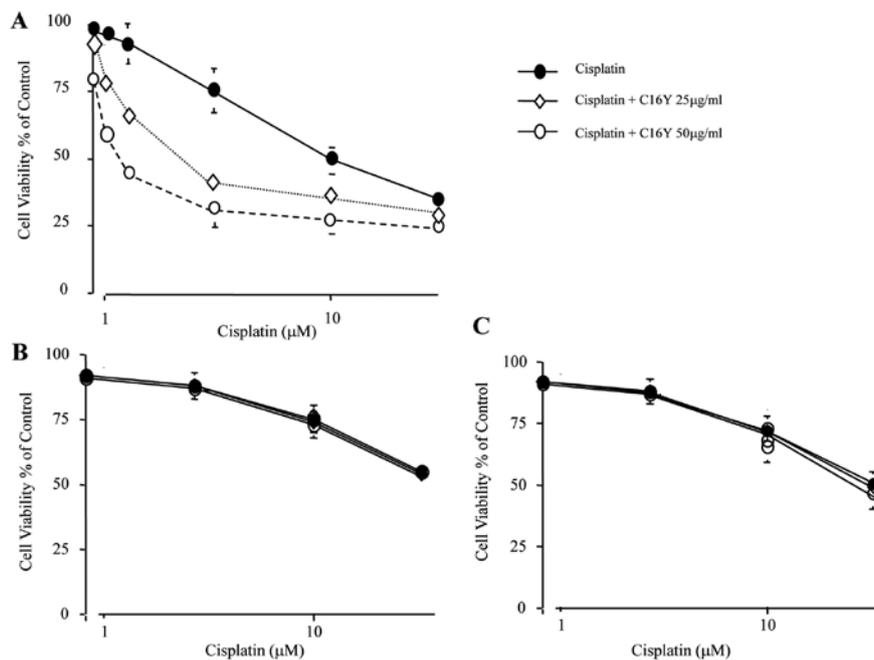

Figure 5. Synergistic effect on apoptosis of the combination drug treatments correlates with loss of cell viability and no elevation in cytotoxicity in the non-transformed human endothelial cells. (A) OVACAR3cells, (B) HUVECs (B and C) NIH/3T3 cells were incubated with 0, 25 and 50 μg/ml of C16Y alone or with combinations of increasing concentrations of cisplatin (from 1-20 μM) for 24 h in DMEM/F12 medium without FCS. At the end of the incubation the MTT assay was performed. Data represent the mean ± SD of three replicates. Values of cisplatin-C16Y (25 and 50 μg/ml)-treated cells are significantly different from cells treated with cisplatin alone at 1-10 μM (P<0.001) in (A) and not significantly different in (B) and (C).

right, compared to the curve in the absence of C16Y. In addition, we found that even at a low concentration of 1 and 3 μM cisplatin, C16Y at 10 and 25 μg/ml increased the incidence of apoptosis in the OVACAR3s by 3-5-fold.

We then investigated the synergistic induction of apoptosis by cisplatin and C16Y (Fig. 4). Cells were incubated at 37°C without FCS for 24 h in the presence of 25 μg/ml C16Y alone,

in the presence of 1 μM cisplatin alone, or with a combination of C16Y and cisplatin at the above concentrations. Cells were fixed and stained for apoptotic nuclei according to the TUNEL method. A low incidence of apoptotic nuclei was observed following 25 μg/ml C16Y alone (Fig. 4B), or 1 μM cisplatin alone (Fig. 4C). However, there was clearly a high incidence of apoptotic nuclei when the OVACAR3s were treated with the
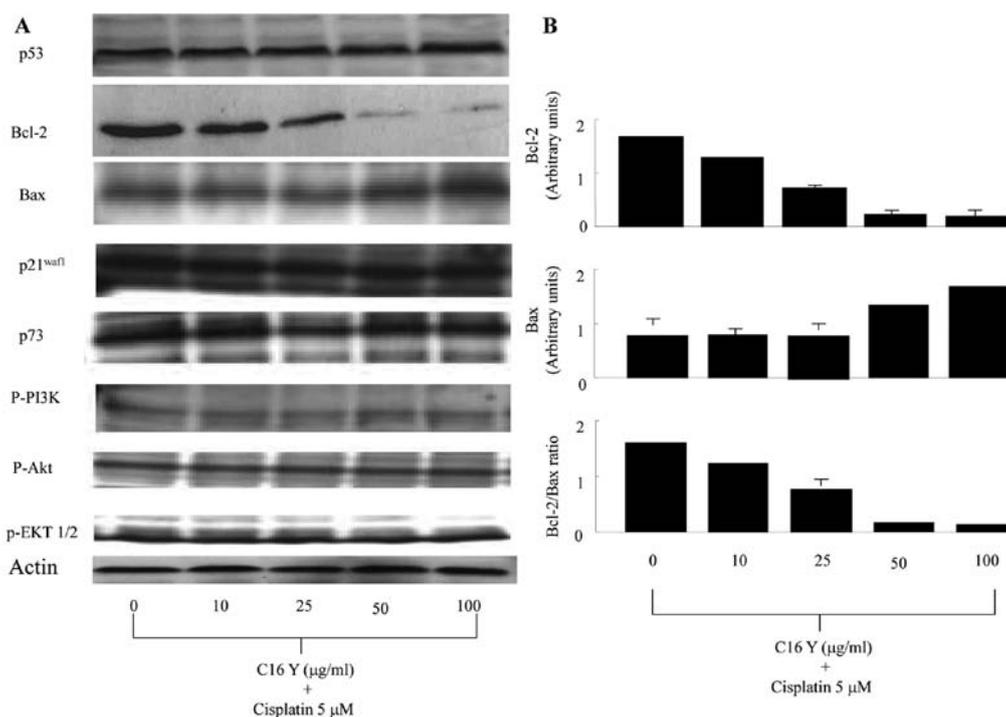
Figure 6. Expression of p53, p21, p73, MAPK, PI3-AKT, Bcl-2 and Bax in controls, C16Y and/or cisplatin-treated OVACAR3 cells. Cells were incubated with each concentration (10, 25, 50 and 100 µg/ml) of C16Y alone or in combination with 5 µM cisplatin for 24 h in DMEM/F12 medium without FCS. Cell lysates were then prepared and Western blot analysis was performed using specific antibodies to p53 (PAb421, directed against human and mouse), p21, p73, phosphorylation of ERK1/2, PI3-AKT, Bax and Bcl-2. (B) Densitometric analysis of Bcl-2 and Bax. Data represent the mean ± SD of three independent measurements. The floatation of the individual measurements from the mean exceeded 5%.

combination of 25 µg/ml C16Y and 1 µM cisplatin (Fig. 4D). Fig. 5A shows the inhibition of cell growth of OVACAR3s by the combination of cisplatin and C16Y in a dose-dependent manner. The combination of the two drugs clearly had a synergistic effect at 1-10 µM cisplatin. In the presence of 25 or 50 µg/ml C16Y (in this range no effect against HUVECs or NIH/3T3 cells was observed), the EC_{50} for cisplatin was reduced to 3.5 or 2.0 µM, respectively. Fig. 5B and C show the inhibition of cell growth in normal cells (HUVEC and NIH/3T3 cells) by both cisplatin and C16Y in a dose-dependent manner. The combination of the two drugs did not induce a synergistic effect in normal cells.

We next examined whether the synergistic effect of these drugs involved changes in the death genes or survival genes. The Bax protein expression levels were markedly increased by combination of C16Y and cisplatin in a dose-dependent manner (Fig. 6). Furthermore, the combination of the two drugs resulted in a further significant reduction in Bcl-2, underscoring the pronounced synergy of cisplatin and C16Y. Co-incubation of OVACAR3s with 5 µM of cisplatin and 50 µg/ml C16Y reduced the Bcl-2/Bax ratio expression by 100-fold, demonstrating a pronounced synergistic effect compared to 5 µM cisplatin alone. On the other hand, C16Y did not activate any other signal transduction pathway, such as p53, p21^{waf1}, p73, ERK1/2 or PI3-AKT.

Discussion

In the present report we have described that C16Y, in the concentration range safe for normal cells and thus with no serious side effects, used in combination with cisplatin can induce massive cell death in ovarian cancer cells. This combination was particularly effective in its synergistic action at concentrations of cisplatin that were much lower than those used for chemotherapy *in vivo* (1). This may minimize the toxic side effects of cisplatin as well as of C16Y (1).

Peptide C16Y is not toxic to normal cells at concentrations at least up to 50 µg/ml, and may interfere with the normal functions of laminin-1, as well as other molecules that use the integrin α5β1 and αvβ3 receptors. Angiogenesis has been shown to require integrin αvβ3 activity (5). Laminin-1 has many biological activities that would promote tumor growth and angiogenesis (2,3), as it promotes cell adhesion, migration, invasion, and protease activity, and laminin-1-adherent cells are more malignant than non-adherent cells or fibronectin-adherent cells. Ponce *et al* (5) indicated that the C16 site on laminin-1 is functionally important *in vivo* in both tumor growth and angiogenesis, and that this active site on laminin-1 is blocked by the C16Y peptide. Thus, there appears to be an important interaction between the αvβ3 and C16Y peptides.

Inhibition of the αvβ3 integrins has previously been shown to reduce tumor growth and metastasis through the disruption of tumor angiogenesis (10-13). Li *et al* (14) have demonstrated that targeting integrins αv and β3 by the technique of anti-sense gene therapy suppresses the growth of hepatocellular carcinomas by inhibiting tumor angiogenesis and inducing cellular apoptosis. In particular, the αv integrins contribute to regulation of the cell cycle and are able to inhibit apoptosis through the p53 pathway. Furthermore, Lin *et al* (15) found that resveratrol, which is a naturally occurring polyphenol, causes apoptosis in cultured cancer cells. They described a cell surface resveratrol receptor on the extracellular domain

of the heterodimeric αvβ3 integrin in MCF-7 human breast cancer cells. This receptor is linked to induction by resveratrol of extracellular-regulated kinases 1 and 2 (ERK1/2)- and serine-15-p53-dependent phosphorylation leading to apoptosis of breast cancer cells.

It has been previously described that cisplatin-induced cytotoxicity involves the induction of apoptosis (1), but the present study is the first to demonstrate the dramatic synergistic effect of cisplatin and C16Y on the induction of apoptosis via a reduction in Bcl-2 and Bax expression, even at 5 μM cisplatin. In this study no significant changes in p53, p21, PI3K-AKT or ERK1/2 expression were observed. Constant expression of Bax and Bcl-2 was detected in the OVACAR3 ovarian cancer cells, suggesting the possibility that the Bax and Bcl-2 ratio plays an important role in C16Y-and-cisplatin- induced apoptosis. However we cannot exclude the possibility that Bcl-xLong, or any other member of the Bcl-2 gene family, is involved in the modulation of apoptosis in this cell system. A more detailed study of all the Bcl-2 and Bax gene family will be performed in the future.

Davidson *et al* (16) reported that the αv- and β1-integrin subunits are frequently expressed in ovarian cancer cells in effusions, and that the αv-integrin subunit is a powerful diagnostic marker for ovarian cancer cells. They concluded that the high expression of integrin subunits, which play roles in binding mesothelium, invasion, and angiogenesis in carcinoma cells in both peritoneal and pleural effusions, suggests that cells at both sites have metastatic potential. Guo *et al* (17) investigated the possible correlation between the expression of integrin αv, β5 and β3 in tumor tissues and the response to chemotherapy and survival of 77 patients with ovarian epithelial carcinoma. They found, in the drug resistant group, that the expression of integrin αv was significantly higher than in the drug sensitive group. According to multivariate analysis, the expression of integrin αv was an independent factor correlated with drug resistance and disease prognosis for patients with ovarian epithelial cancer. This data suggests that the inhibition of the αvβ3 integrins is important to overcome advanced or drug-resistant ovarian cancer.

The combination of C16Y and cisplatin used in the present study exhibited almost no cytotoxicity to the HUVECs or fibroblasts. Thus, we believe that the effects of combined treatment with C16Y and cisplatin, targeting integrins αv and β3, may be mediated in part by their ability to down-regulate the Bcl-2/Bax ratio in ovarian cancer cells, in particular in drug resistance and aggressiveness of ovarian cancer. These results suggest that this combined treatment should be examined in other ovarian cancer cells and also in an *in vivo* animal experimental model.

Acknowledgements

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