Efficient inhibition of cisplatin-resistant human ovarian cancer growth and prolonged survival by gene transferred vesicular stomatitis virus matrix protein in nude mice

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Background: The vesicular stomatitis virus matrix protein (VSVMP) has been receiving attention as an anticancer agent because of its ability of inducing apoptosis.

Materials and methods: Nude mice bearing A2780s and A2780cp ovarian tumors were treated twice weekly with i.v. administration of 50 µg VSVMP/250 µg liposome complex, 50 µg empty plasmid/250 µg liposome complex, 0.9% NaCl solution or weekly with i.p. administration of cisplatin (5 mg/kg) for 3 weeks. Tumor volume and survival time were observed. TUNEL assay and CD34 vessel staining were conducted in tumor tissue. Antiangiogenesis in vivo were determined by sponge assay. Antiproliferative and apoptosis-inducing activities of VSVMP in vitro were tested on MS1murine endothelial cells and four human ovarian cancer cell lines: A2780s, A2780cp, HO8910 and COC1.

Results: Administration of VSVMP resulted in significant inhibition (87%–98% maximum inhibition relative to controls) in the growth of A2780s and A2780cp tumor xenografts, and prolonged the survival of the treated mice. Complete tumor regression happened in VSVMP-treated mice in both tumor models. These antitumor responses were associated with marked increases in tumor apoptosis and reductions in intratumoral microvessel density.

Conclusions: Our data indicate that VSVMP may provide an effective approach to inhibit both cisplatin-sensitive and -resistant human ovarian cancer growth with minimal side-effects.

Key words: angiogenesis, apoptosis, cisplatin-resistant, ovarian cancer, vesicular stomatitis virus matrix protein (VSVMP)

Introduction

Epithelial ovarian cancer is the number one cause of death from gynecologic malignancy. Except for some improvement in survival length with the introduction of platinum and paclitaxel therapy, the long-term survival remains poor due to eventual tumor recurrence and emergence of drug-resistant disease. This dismal status quo provides a strong stimulus for developing new therapeutic approaches, particularly for agents which lack cross-resistance with established compounds [1].

Vesicular stomatitis virus (VSV), the prototype virus of the Rhabdoviridae, can preferentially replicate in immortalized and malignant cells and finally induce apoptosis [2, 3]. Several in vivo studies have demonstrated the growth-inhibiting effect of VSV in various tumor models [3–5]. However, the application of VSV is limited by the potential biohazard of virus infection. The vesicular stomatitis virus matrix protein (VSVMP), one of the five structural proteins (N, P, M, G and L) of this virus, causes considerable cytopathogenesis of VSV in the absence of other viral components. The mechanisms by which the matrix protein induces cytopathic effects are disruption of all three types of cytoskeletal elements, including actin, vimentin, and tubulin by interacting with them [6, 7], and general inhibition of host cell gene expression [3, 8, 9], which can result in the systemic breakdown of the cell by apoptosis [10, 11].

Our laboratory has demonstrated previously the efficient inhibition of i.p. SKOV3 human ovarian cancer growth by i.p. administering a recombinant plasmid DNA carrying VSVMP-cDNA using cationic liposome as gene delivery system.
Considering the different mechanism of VSVMP from chemotherapeutic agents such as cisplatin, the present study was aimed to find out whether it could overcome cisplatin resistance in ovarian cancer. We tested the VSVMP on A2780s and A2780cp human ovarian cancer cells. Different from the previous study, we used s.c. tumor model and changed the way of administration into i.v. administration. The VSVMP demonstrated a profound inhibiting effect on the growth of not only cisplatin-sensitive but also cisplatin-resistant ovarian cancer cells in vitro and in vivo, and significantly prolonged the survival of the treated mice.

**materials and methods**

**cell culture and reagents**

The derivation and source of established human ovarian cancer cell lines A2780s and A2780cp have been described previously [13]. SV40 T antigen immortalized murine endothelial cell line (MS1) was obtained from American Type Culture Collection. Ho8910 ovarian cancer cell line was obtained from the Institute of Cell Biology in Shanghai. COC1 human ovarian cancer cell line was obtained from China Center for Type Culture Collection. Cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin.

Cisplatin (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO (Sigma Chemical) and diluted in NaCl solution (NS) before use. The final concentration of DMSO was 0.1%.

**plasmid construction and liposome preparation**

The plasmid construction and liposome preparation were done as previously described [12]. The plasmid carrying VSVMP-cDNA was also named VSVMP and the empty plasmid pcDNA3.1 was used as a control (e-p).

**cell proliferation assay**

Ovarian cancer cells \((2 \times 10^5)\) were grown in six-well plates and incubated for 24 h to 30% confluence. DNA (VSVMP or e-p)/liposome complexes were prepared in DMEM medium without serum, which contained 2 µg DNA/10 µg liposome and left at room temperature for 30 min, NS was also used as a control agent. Cells were incubated with the above agents for 6 h, the medium was then changed by 2 ml of DMEM supplemented with FCS and incubated for additional 24–72 h. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage of FCS and incubated for additional 24–72 h. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage of inhibition% cells was determined by the log-rank test; \(P < 0.05\) was defined as significant.

**apoptosis assays and cell cycle analysis**

A2780s, A2780cp, Ho8910 and COC1 ovarian cancer cells were treated with VSVMP, e-p or NS for 48 h. At the same time, A2780s and A2780cp cells were treated with cisplatin (10 µM or 100 mM) for 48 h. Quantitative evaluation of cellular apoptosis and cell cycle analysis were carried out by flow cytometric analysis using PI staining method. Morphological changes of VSVMP-treated cells were observed by PI staining fluorescence microscopy. DNA fragmentation assay was done as previously described [15]. Images of cells were taken by using Zeiss Axiovert 200 microscope and AxioCam MRm camera.

**in vivo animal treatment protocol**

Female athymic BALB/c nude mice, 6–8 weeks old, were maintained in pathogen-free conditions and fed sterile chow. A2780s cells or A2780cp cells \((2 \times 10^6)\) in 0.1 ml of PBS were injected s.c. into the right flank of each mouse. Mice were randomly assigned into one of the following four groups \((n = 5)\): (i) mice treated with 50 µg VSVMP/250 µg liposome complexes, (ii) mice treated with 50 µg e-p/250 µg liposome complexes, (iii) mice treated with 100 µl of 0.9% NS and (iv) mice treated with cisplatin (5 mg/kg).

Before i.v. administration, the DNA/liposome mixture was gently mixed and incubated at room temperature for 30 min. Treatment (twice per week) started when tumors were palpable and continued for 3 weeks. To investigate cisplatin resistance of A2780cp tumors, 5 mg/kg of cisplatin (Qilu Pharmaceutical Co., Shandong, China) was administered i.p. once per week for 3 weeks. Tumor sizes were measured every 3 days using the formula \(A \times B^2 \times 0.5236\) (A, length; B, width; all measured in millimeters).

**evaluation of possible adverse effects**

For mice treated with VSVMP, gross measures such as weight loss, ruffling of fur, life span, behavior and feeding were investigated in particular for potential toxicity. Tissues of heart, liver, spleen, lung, kidney, brain, pancreas, bone marrow and intestine were also fixed in 10% buffered formalin solution and embedded in paraffin. Sections of 3–5 µm were stained with hematoxylin and eosin (H&E).

**histologic analysis**

Analysis of apoptotic cells within tumor tissue was done by using a commercially available TUNEL kit (Promega, Madison, WI), following the manufacturer’s protocol. Staining for microvessel was done as previous described [16]. Images of tumor tissue and microvessels were taken by using Olympus BX60 microscope and Spot Flex camera.

**in vivo sponge angiogenesis assay**

The sponge angiogenesis assay was done as previously described [17]. Briefly, VEGF (PeproTech Inc., Rocky Hill, NJ) was admixed with Matrigel (Becton Dickinson, Bedford, MA) before adding to surgical sponges (4 x 4 x 2 mm). Each sponge was implanted s.c. on the abdomen of female athymic mouse. Mice (five per group) were treated twice weekly with i.v. administration of 50 µg VSVMP/250 µg liposome complex, 50 µg e-p/250 µg liposome complex or 0.9% NS. Two weeks later, sponges were harvested and homogenized in 0.1 ml ddH2O. Lyses were transferred to a 96-well plate and hemoglobin content was quantified by measuring the colorimetric change following addition of tetramethylbenzidine. The reaction was stopped by adding an equal volume of 0.09 N H2SO4. Inhibition was calculated as a percentage of angiogenesis of the NS-treated sponges.

**statistical analysis**

Comparisons of tumor volume among different groups were carried out by using one-way analysis of variance. Comparison of angiogenesis inhibition in sponge assay was carried out by using Student’s t-test. Survival curves were on the basis of the Kaplan–Meier method and statistical significance was determined by the log-rank test; \(P < 0.05\) was defined as significant.

**results**

**VSVMP induced cell growth inhibition in ovarian cancer cells**

A2780s, A2780cp, HO8910 and COC1 cells were assayed for cell proliferation after treatment with VSVMP. As shown in Figure 1A, VSVMP (2 µg DNA/10 µg liposome) caused a time-dependent inhibition of cell growth in the four ovarian cancer cell lines. By contrast, treatment with e-p (2 µg DNA/10 µg liposome) had no effect.
VSVMP induced apoptosis in ovarian cancer cells

To evaluate whether the antiproliferative effect of VSVMP was associated with apoptosis induction, we employed three different methods to detect apoptotic cells: flow cytometry, morphological analysis and DNA fragmentation assay. From the results of flow cytometry, we found that the apoptotic cells (sub-G1 cells) among A2780cp cells only accounted for 22.3% when treated with 100 μM cisplatin, while the percentage was 50.8 in A2780s cells when treated with 10 μM cisplatin. So, the A2780cp cells were at least 10 times more resistant to cisplatin than the A2780s cells. In A2780s tumor model, the apoptotic cells accounted for 44.6% in VSVMP-treated group versus 13.7% in e-p-treated group and 6.2% in NS-treated group. In A2780cp tumor model, the apoptotic cells accounted for 46.1% in VSVMP-treated group versus 11.6% in e-p-treated group and 5.1% in NS-treated group (Figure 1B). Similar results were obtained in Ho8910 and COC1 ovarian cancer cells (Figure 1C). VSVMP-treated cells resulted in morphological changes characterized as apoptosis: a brightly red fluorescent condensed nuclei (intact or fragmented) and apoptotic bodies. However, these changes seldom happened in e-p-treated or NS-treated cells (Figure 2A). In agarose gel electrophoresis of VSVMP-treated cells, there was a ladder-like pattern of DNA fragments consisting of 180–200 base pairs, which was consistent with internucleosomal DNA fragmentation (Figure 2B). Results obtained in flow cytometry strongly
correlated with DNA fragmentation assay and morphological changes in fluorescence microscopy of PI staining.

Cell cycle analyses were also conducted on A2780s and A2780cp cells. After 48 h of VSVMP treatment, the number of G0/G1, G2/M and S phase cells decreased among the A2780s cells and the number of G0/G1 and S phase cells decreased among the A2780cp cells, when sub-G1 cells increased in number, compared with NS or e-p-treated cells (Figure 2C). A similar pattern of cell cycle perturbation by VSVMP was found in Ho8910 and COC1 ovarian cancer cells (data not shown). It thus seems likely that the inhibition of cell proliferation by VSVMP may in part be a result of apoptotic cell death.

VSVMP inhibited both A2780s and A2780cp human ovarian cancer xenografts in nude mice
In A2780s tumor model, there was a significant difference in tumor volume between VSVMP-treated mice and control therapies. Mean tumor volume (±standard deviation (SD)) in VSVMP-treated mice was 212 ± 391 versus 1636 ± 378 m³ in e-p-treated mice, 3242 ± 877 m³ in NS-treated mice and 2924 ± 684 m³ in cisplatin-treated mice, P < 0.01; Figure 3B. Complete tumor regression happened in all of the mice treated with VSVMP, only one of the tumors reappeared 1 week after cessation of treatment.

VSVMP prolonged the survival of tumor-bearing mice
Survival of the tumor-bearing mice treated with VSVMP in both tumor models was significantly prolonged compared with the control therapies, P < 0.01. There was no significant difference in survival time between VSVMP-treated mice and cisplatin-treated mice in A2780s tumor model. Mice with complete tumor regression gained long-time survival in VSVMP-treated groups of both tumor models (Figure 3C, D).

toxicity observation
No gross abnormalities were found in VSVMP-treated mice. Furthermore, H&E histological staining of the liver, lung, kidney, spleen, brain, heart, pancreas, intestine and bone marrow observed by two pathologists did not reveal any significant differences between VSVMP-treated and the control mice.
increased intratumoral apoptosis in VSVMP-treated mice

In order to study the antitumor mechanism *in vivo*, TUNEL assay was carried out. Many strongly positive nuclei identified as apoptosis were found in VSVMP-treated tumor tissue, whereas such nuclei were rare in e-p-treated or NS-treated groups (Figure 4).

inhibition of angiogenesis by VSVMP

Angiogenesis within tumor tissue was assessed by CD34 immunohistochemical vessel staining. Administration of VSVMP resulted in the apparent inhibition of angiogenesis in both A2780s and A2780cp tumors compared with the control therapies. There were plenty of apoptotic cancer cells and destroyed vessels within tumor tissue of VSVMP-treated mice,
and apoptosis of cancer cells occurred in proximity to destroyed vessels. However, these vessels could hardly be stained by CD34 staining because their endothelium was destroyed (Figure 5A).

The inhibition of angiogenesis was confirmed by sponge angiogenesis assay. Following 2 weeks of dosing, VSVMP sufficiently inhibited VEGF-induced angiogenesis by 71.7% relative to NS-treated animals, while the inhibition rate of e-p administration was 15.7%, $P < 0.01$ (Figure 5B).

apoptosis induction of VSVMP on MS1 murine endothelial cells in vitro

In order to find out the possible mechanism by which the VSVMP inhibited angiogenesis, we tested VSVMP on SV40 T antigen immortalized murine endothelial cells in vitro. Cells treated with VSVMP resulted in morphological changes characterized as apoptosis and demonstrated a ladder-like pattern of DNA fragments. Flow cytometric analysis revealed that there were a large amount of apoptotic cells among VSVMP-treated cells compared with e-p-treated or NS-treated cells (Figure 6).

**discussion**

Drug resistance has made epithelial ovarian cancer the most lethal gynecologic malignancy [18]. It has been reported that the acquired drug resistance of ovarian cancer cell is associated with alterations in apoptosis [19]; inhibition of apoptosis is taken to be a major contributing factor to cisplatin resistance in various ovarian cancer cell lines [20, 21]. So, patients with resistant ovarian cancer are often switched to another active agent, presumably having a different mechanism of apoptosis induction.

The VSVMP has been receiving attention as an anticancer agent because of its ability of inducing apoptosis. The mechanisms by which the matrix protein induces apoptosis are disorganization of cytoskeletal elements [6, 7] and general inhibition of host cell gene expression [3, 8, 9]. While the effect of cisplatin appears to result from inhibition of replication by cisplatin–DNA adducts and subsequent induction of p53-dependent apoptosis [22], which is totally different from mechanisms of VSVMP. In the current study, we tested the VSVMP on cisplatin-sensitive and -resistant human ovarian cancer models. VSVMP efficiently inhibited the

![Figure 5](image-url)

**Figure 5.** Inhibition of angiogenesis by vesicular stomatitis virus matrix protein (VSVMP). (A) Tumor angiogenesis was assessed by immunohistochemical staining with anti-CD34 antibody (brown) on paraffin-embedded sections. Representative sections were taken from A2780s and A2780cp tumor tissue of NaCl solution (NS)-treated, e-p-treated and VSVMP-treated mice. Original magnification ×400. (B) *In vivo* evaluation of antiangiogenesis using the sponge model. Following 2 weeks of dosing (50 μg DNA/250 μg liposome complex administered i.v. twice per week), VSVMP sufficiently inhibited VEGF-induced angiogenesis by 71.7% relative to NS-treated nude mice, while the inhibition rate of e-p administration was 15.7%. *$P < 0.01$. Columns: average of five mice; bars: ±standard deviation.
growth of both A2780s and A2780cp cancer xenografts and prolonged the survival of the treated mice, without causing any gross toxicity of the animals. These results strongly indicate that VSVMP may overcome cisplatin resistance at least partly in some tumors. Although we did not examine the synergism of VSVMP with cisplatin, it should be confirmed by in vivo experiments in future with other cell lines.

The reason why we choose VSVMP instead of VSV itself is the limitations of VSV therapy. Infection of VSV in human beings may cause a mild febrile illness in some cases [23], which implies that application of replicating-competent VSV should be cautious due to its potential biohazard. Furthermore, VSV infection eventually triggers humoral and cellular immune responses against VSV itself within 1 or 2 weeks [24–26], which makes the cancer treatment incomplete. Therefore, the application of VSVMP is the best solution.

We had also made several observations concerning the antitumor mechanism of VSVMP on various ovarian cancer cells in vitro, including cell proliferation assay, flow cytometric analysis of apoptotic cells and cell cycle, PI staining fluorescence microscopy and DNA fragmentation assay. TUNEL assay was conducted in tumor tissue in vivo. Our findings were consistent with the apoptosis-inducing ability of VSVMP reported before [10, 11].

Another significant observation was that VSVMP could inhibit tumor angiogenesis. Angiogenesis plays a vital role in tumor growth and metastasis [27, 28], which can be inhibited by endothelial cell apoptosis [29, 30]. In the current study, VSVMP induced apoptosis of SV40 T antigen immortalized murine endothelial cells in vitro and decreased the number of intratumoral microvessels in vivo. We had also observed that there were plenty of apoptotic cancer cells and destroyed vessels within tumor tissue of VSVMP-treated mice, and apoptosis of cancer cells occurred in proximity to destroyed vessels. The antiangiogenic effect of VSVMP was further confirmed by sponge angiogenesis assay. Our findings may indicate that VSVMP inhibits angiogenesis through endothelial cell apoptosis.

In conclusion, we demonstrated that the VSVMP had potent activity against human ovarian cancer cells in vitro and in vivo by triggering apoptosis of cancer cells and inhibiting angiogenesis through endothelial cell apoptosis. Because of the different mechanisms from platinum compounds, this drug was effective in cisplatin-resistant ovarian cancer. Given the strong antitumor effect and minimal toxicity, VSVMP has potential as a novel therapeutic agent for the treatment of ovarian cancer, particularly for patients with cisplatin resistance.

Figure 6. Vesicular stomatitis virus matrix protein (VSVMP) induced apoptosis of SV40 T antigen immortalized murine endothelial cells in vitro. MS1 cells were treated with NaCl solution (NS), e-p or VSVMP. (A) DNA fluorescence histograms of PI-stained cells and morphological changes of cells (48 h after transfection). Original magnification ×200. (B) DNA fragmentation of VSVMP-treated cells (72 h after transfection): cells were treated with NS (lane a), e-p (lane b) or VSVMP (lane c).
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