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Efficient inhibition of intraperitoneal human ovarian cancer growth and prolonged survival by gene transfer of vesicular stomatitis virus matrix protein in nude mice

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Abstract

Objective. Vesicular stomatitis virus (VSV) matrix protein (MP) has been reported to be capable of inducing apoptosis *in vitro* in the absence of other viral components. In the present study, the antitumor effect of a recombinant plasmid encoding VSVMP on human ovarian cancer and its apoptosis-inducing efficacy *in vivo* were further investigated.

Methods. The recombinant plasmid DNA carrying VSVMP-cDNA (VSVMP-p) was constructed. SKOV3 ovarian cancer cells were transfected with VSVMP-p and examined for apoptosis by Hoechst 33258 staining and flow cytometric analysis. For *in vivo* study, intraperitoneal ovarian carcinomatosis models in nude mice were established and randomly assigned into four groups to receive six twice-weekly i.p. administrations of VSVMP-p/liposome complexes, empty plasmid/liposome complexes, liposome alone or 0.9% NaCl solution, respectively. The weight of intraperitoneal carcinomatosis and the survival were monitored. Tumor tissues were inspected for apoptosis by TUNEL and Hoechst-33258 assay.

Results. Plentiful apoptosis were observed in SKOV3 cells transfected with VSVMP-p. VSVMP-p reduced intraperitoneal tumor weight by about ~90% compared with control agents ($p<0.01$) and significantly prolonged the survival of tumor-bearing mice ($p<0.05$), with *in vivo* apoptosis index of 12.6±2.7% which was much higher than that of control groups (<4%) ($p<0.05$). Interestingly, this antitumor effect was accompanied by a noticeable NK cell accumulation. The treatment with VSVMP-p was devoid of any conspicuous toxicity.

Conclusions. These observations suggest that VSVMP-p have strong antitumor effects by inducing apoptosis and possibly NK cell-mediated tumor resistance mechanisms, and it may be a potentially effective novel therapy against human ovarian cancer.

Keywords: Vesicular stomatitis virus matrix protein (VSVMP); Apoptosis; Lymphocyte accumulation; Ovarian cancer; Cationic liposome

Introduction

Vesicular stomatitis virus (VSV), a negative-stranded RNA rhabdovirus with a single nonsegmented genome that contains only five protein-encoding genes (N, P, M, G and L), can preferentially replicate in immortalized and malignant cells and eventually induce apoptosis [1–4]. Studies by a number of researchers have also confirmed that VSV can suppress the growth of various tumors when introduced intratumorally or intravenously in tumor-bearing animal models [3–7]. The intriguing questions for researchers are which viral components play an important role in inducing apoptosis in VSV-infected cells and whether they still possess this function when used alone to avoid potential biohazard of virus infection. Recent studies have demonstrated that it is the matrix protein (MP), a structural component of the virion, that causes considerable cytopathogenesis of VSV in the absence of other viral components, including the inhibition of host gene expression, the elicitation of cell rounding and the induction of apoptosis [8–13]. Kopecky and Lyles [14] further analyzed the cause and effect relationships of the three cellular effects of MP and

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concluded that inhibition of host gene expression caused apoptosis, which in turn led to the induction of cell rounding.

The potent ability of MP to induce apoptosis *in vitro* raises such hypotheses as "Whether MP can elicit apoptosis of tumor cells *in vivo*" and "Can it be a promising agent for malignant disease". To test these hypotheses, we constructed a recombinant plasmid DNA carrying VSVMP-cDNA (VSVMP-p), administered it in a few tumor models using cationic liposome as a gene delivery system (including Lewis lung carcinoma and CT26 mouse colon adenocarcinoma *in vitro*, and s.c. Lewis lung carcinoma model and s.c. MethA fibrosarcoma model *in vivo*) and found that it had potent antitumor activity (Zhao JM et al., manuscript submitted for publication).

Ovarian cancer accounts for 4% of all cancers in women and is the principal cause of death among the gynecological malignancies [15]. Aggressive surgical debulking along with multiagent cytotoxic chemotherapy has therapeutically benefited patients with advanced, epithelial ovarian cancer and newly developed biotherapies along with rarely used radiation therapy may be helpful supplements. However, the mortality rate of ovarian cancer remains exceptionally high [16,17]. This dismal status quo reflects a current lack of available, more effective treatments. Considering that gene transfer of VSVMP was able to induce apoptosis *in vitro* and inhibit the growth of a few tumors *in vivo*, the present study is designed to evaluate the antitumor effect of VSVMP-p as an experimental treatment for ovarian cancer. In this study, VSVMP-p proved able to effectively induce apoptosis of SKOV3 ovarian cancer cells *in vitro*; furthermore, intraperitoneal administration of VSVMP-p/liposome complexes demonstrated noticeable efficacy against peritoneal carcinomatosis model in nude mice, resulting in suppressed tumor growth and prolonged survival owing to the capacity of VSVMP-p to induce apoptosis and possible NK cell accumulation.

Materials and methods

Cell line

The human ovarian serous cystadenocarcinoma cell line SKOV3 was obtained from American Type Culture Collection (Rockville, Maryland) and cultured in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 0.1 mg/ml amikacin. Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and passaged every 5 days at a split ratio of 1:4 using trypsin.

Plasmid construction and liposome preparation

pcDNA3.1 plasmid (Invitrogen, San Diego, CA) expressing wild-type VSVMP, named VSVMP-p, was constructed in our laboratory. Briefly, cDNA clone encoding VSVMP was PCR amplified with the upstream primer CGC GGA TCC ATC ATG AGT TCC TTA AAG AAG and the downstream primer CGG AAT TCT CAT TTG AAG TGG CTG ATA GAA TCC. Then it was digested with *Bam*H1/*Eco*R1 and inserted into pcDNA3.1 digested with *Bam*H1/*Eco*R1 to generate VSVM-p. As a control, pcDNA3.1 plasmid without VSVMP-cDNA was used as an empty vector (e-p). Colonies of *Escherichia coli* containing VSVMP-p or e-p were cultured in Luria-Bertani broth containing 100 μg of ampicillin/ml. Large-scale plasmid DNA was purified using an EndoFree Plasmid Giga kit (Qiagen, Chatsworth, CA). The DNA was eventually dissolved in sterile endotoxin-free water at a concentration of 5.0 mg/ml, stored at −20°C before use. The recombinant VSVMP-p was confirmed by restriction digestion and DNA sequencing.

The lipids DOTAP (dioleyl trimethylammonium propane; Avanti Polar Lipids Inc., AL) and DOPE (dioleyl phosphatidylethanolamine; Avanti Polar Lipids Inc., AL) (1:1 molar ratio) were dissolved in chloroform supplemented with methanol (3:1 volume ratio). The resulting mixture was dried in a rotary 100-ml round-bottomed flask and organic solvent was further removed under vacuum for 2 h. The lipid film was hydrated in appropriate amount of sterile water to yield a final concentration of 2.5 mg/ml. Then the liposome solution was vortexed for 1 min, sonicated for 10 min to form small unilamellar vesicles and stored at 4°C.

Apoptosis assay in vitro

In order to determine the optimal plasmid:liposome ratio (μg/μg) for efficient gene delivery, a series of experiments *in vitro* with different plasmid: liposome ratios transfecting SKOV3 cells were carried out. The plasmid used herein was the recombinant pcDNA3.1 plasmid encoding for the green fluorescent protein (GFP) and a maximum expression was obtained when 5 μg liposome/μg DNA was used (data not shown).

To test the effect of VSVMP-p *in vitro*, aliquots of 1×10^5 SKOV3 cells were grown in each well of 6-well plates in triplicate and incubated for 72 h to 80% confluence. DNA (VSVMP-p or e-p)/liposome complexes were prepared in RPMI 1640 medium, which contained 2 μg DNA and 10 μg liposome, and left at room temperature for 30 min. In addition, 10 μg liposome alone and medium alone were also used as control agents. The cells were incubated with the above agents for 6 h, rinsed three times with PBS and then 1.5 ml of RPMI 1640 supplemented with FCS was added to each well, with a continued incubation for an additional 48 h. Apoptosis was observed by Hoechst 33258 staining (Apoptosis-Hoechst staining kit; Beyotime Biotechnology, Chin). Briefly, cells were immersed in 0.5 ml of methanol for 15 min, followed by rinse with PBS twice. Then cells were stained with 1 μg/ml Hoechst 33258 compounds in a dark chamber at room temperature for 10 min and rinsed twice in PBS again. Cells were analyzed by fluorescence microscopy using excitation 348 nm/emission 480 nm wavelengths. The apoptotic cells are featured as pyknotic and fragmented nuclei emitting intense fluorescence [18].

In addition, quantitative evaluation of cellular apoptosis was performed by flow cytometric analysis using annexin V-propidium iodide (PI) double staining method. Briefly, after processed in 6-well plates as described above, the floated cells were discarded and the attached cells were trypsinized and thereafter washed twice with cold PBS. Cells were resuspended in prediluted binding buffer and stained with FITC-annexin V (BD Pharmingen, CA) for 10 min in the dark at room temperature. Then they were washed and resuspended in binding buffer. PI (1 μg/ml) was added, and the mixtures were analyzed by flow cytometry immediately.

Animals and the establishment of peritoneal carcinomatosis model

Female athymic BALB/c nude mice, 6 to 8 weeks old, were housed in autoclaved microisolator cages in an air-filtered laminar flow cabinet and were given food and water *ad libitum*. All procedures were performed under sterile conditions in a laminar flow hood. This animal experiment was approved by the Institutional Animal Care and Use Committee and was in compliance with all regulatory guidelines.

SKOV3 cell suspension (5×10^6 cells in 100 μl of RPMI 1640 medium) was severally injected s.c. in the backs of five nude mice. The purpose of developing s.c. tumors was to generate histologically intact tumors for i.p. implantation. When the diameter of tumors reached up to about 1 cm, tumors were collected, excised free of necrotic areas and then minced with scissors and tweezers into small particles with a diameter of less than 1 mm. Sufficient RPMI 1640 medium was added into these tumor particles and the total volume of the mixture was made at 16 ml. Then 32 nude mice were respectively inoculated i.p. with 0.5 ml of the mixture in the right lower quadrant with a 14-gauge needle. In a previous study performed in our laboratory, consecutive monitor of the mice undergoing the above procedure revealed that the i.p. inoculation resulted in extensive dissemination of intraperitoneal carcinomatosis and macroscopic or