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BRIEF ARTICLE

Preclinical evaluation of herpes simplex virus armed with granulocyte-macrophage colony-stimulating factor in pancreatic carcinoma

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Abstract

AIM: To investigate the therapeutic efficacy and mechanisms of action of oncolytic-herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) in pancreatic carcinoma.

METHODS: Tumor blocks were homogenized in a sterile grinder in saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly assigned into four groups: a control group, a high dose $HSV^{GM\text{-}CSF}$ group [1×10^7 plaque forming units (pfu)/tumor], a medium dose $HSV^{GM\text{-}CSF}$ group (5×10^6 pfu/tumor) and a low dose $HSV^{GM\text{-}CSF}$ group (5×10^5 pfu/tumor). After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animal by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution. The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor. Furthermore, GM-CSF protein levels in serum were assayed by enzyme-linked immunosorbent assays at 1, 2, 3 and 4 d after injection of $HSV^{GM\text{-}CSF}$.

RESULTS: Injection of the recombinant mouse HSV encoding GM-CSF resulted in a significant reduction in tumor growth compared to the control group, and dose-dependent effects were observed: the relative tumor proliferation rates of the low dose, medium dose and high dose groups on 15 d after injection were 45.5%, 55.2% and 65.5%, respectively. The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively. Furthermore, the production of GM-CSF was significantly increased in the mice infected with $HSV^{GM\text{-}CSF}$. The increase in the GM-CSF level was more pronounced in the high dose group compared to the other two dose groups.

CONCLUSION: Our study provides the first evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer. The enhanced GM-CSF expression might be responsible for the phenomenon.

Key words: Pancreatic carcinoma; Gene therapy; Animal test; Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor

Core tip: Herpes-simplex-virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an engineered oncolytic virus. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and the

ICP47 gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF. Our study provides the first evidence that HSV^{GM-CSF} could inhibit the growth of pancreatic cancer in a dose-dependent manner. Enhanced GM-CSF expression might be responsible for the phenomenon.

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INTRODUCTION

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30% and nearly all patients die from their disease within 7 years of surgery[1,2]. More than 80% patients are unsuitable for radical resection. Further more, it is insensitive to current chemotherapy, radiotherapy and immunotherapy.

Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor (HSV^{GM-CSF}) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers have been completed, providing preliminary data about its safety and effectiveness[3-7]. However, there is little data for pancreatic cancer.

Therefore, we conducted a preclinical evaluation of effects of HSV^{GM-CSF} on pancreatic cancer and explored the mechanisms that may be involved in any antitumor response.

MATERIALS AND METHODS

Experimental chemical

The OrienGene Biotechnology Ltd. (Beijing, China) provided the mouse recombinant GM-CSF herpes simplex virus (HSV^{GM-CSF}) (OrienX010).

Experimental cell and animals

Panc-2 cells: All cells used in this study represent mouse pancreatic carcinoma cell lines. Panc-2 cells were grown in Dulbecco's modification of Eagle's medium.

Animals: Female C-57B mice (4-6 wk, 16-18 g) were provided by the Experimental Animal Center, Peking Union Medical College. The Committee of Animal Care and Use of the university approved the experimental protocol, which met the regulatory requirements of Tumor Hospital, Chinese Academy of Medical Science for the use of experimental animals. All mice were bred in a standard environment and were provided with free access to food and water.

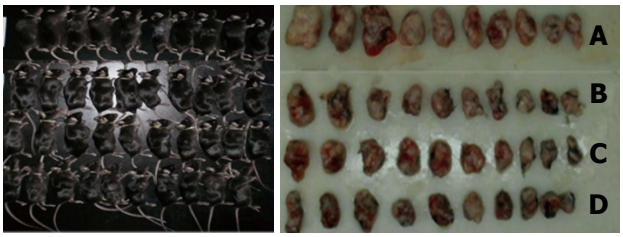

Figure 1 Inhibition of herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor on the proliferation of PANC-2 pancreatic carcinoma xenografts in mice. A: Control group; B: High dosage; C: Middle dosage; D: Low dosage. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated.

Experimental procedure

Injection of transplanted tumors and drug administration in mice followed standard methods used internationally. The Discussion Draft of Guidance Principles of Pharmacodynamics of Antitumor Drugs[8] and Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval[9] were used for guidance. Panc-2 cells were first recovered and amplified for collection of tumor cells, of which a total of 1×10^7-1×10^8 plaque forming units (pfu) virus were subcutaneously injected into each mouse. When the resulting tumor had grown to 2-3 cm in diameter, the tumor tissue was dissected under sterile conditions and cut into blocks of 2 mm^3 with sterile scissors. The tumor blocks were homogenized in a sterile grinder with normal saline. The homogenate was injected into the right armpit of each mouse. After vaccination, the mice were randomly grouped for intratumoral administration of drugs. After initiation of drug administration, body weights and tumor diameters were measured every 3 d. Fifteen days later, after decapitation of the animals by cervical dislocation, each tumor was isolated, weighed and stored in 10% formaldehyde solution (Figure 1). The drug effectiveness was evaluated according to the weight, volume and relative volume change of each tumor.

GM-CSF quantification by enzyme-linked immunosorbent assay

In vivo blood collected by tail vein bleed was centrifuged, and serum was collected and stored at -20 ℃. Mouse GM-CSF concentration was determined by an enzyme-linked immunosorbent assay (ELISA) (Abcam Inc, MA, United States), according to manufacturer's protocol, for cells infected with 1×10^7, 5×10^6 and 5×10^5 pfu/mL.

Animal grouping and drug administration

Three days after vaccination of tumors, the mice were randomly divided into groups with the weights of the

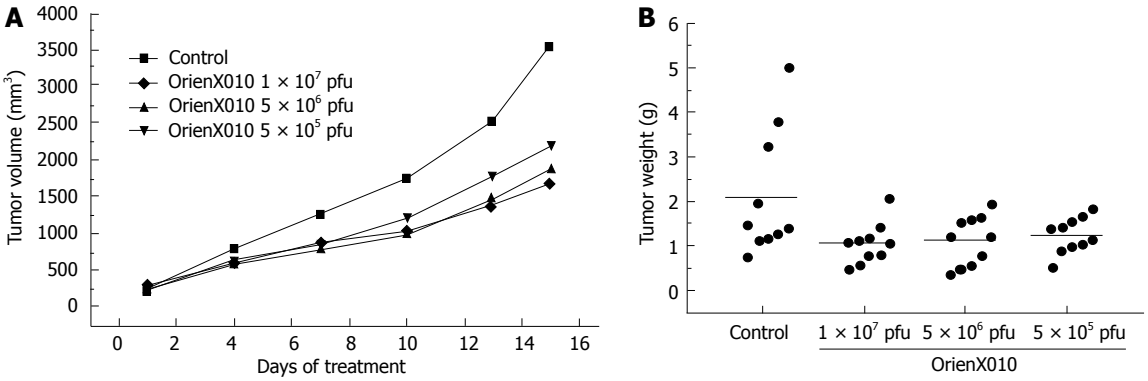

Figure 2 Effect of OrienX010 on the tumor volume (A) and tumor weight (B) of PANC-2 pancreatic carcinoma xenografts in mice. pfu: Plaque forming units.

Table 1 Inhibition of OrienX010 on the growth of PANC-2 pancreatic carcinoma xenografts in mice

Administration	Host (mice) reaction		Tumor reaction					
	Animal number beginning/end	Body weight, g (mean ± SD) beginning/end	Tumor weight, g (mean ± SD)	*Z*	Tumor volume (mm^3)	*J*	RTV	T/C (%)
	10/10	16.5 ± 1.2/19.8 ± 1.9	2.10 ± 1.41		3555.8 ± 1849.8		15.4 ± 5.7	
Tumor injection × 4	10/10	16.4 ± 0.9/18.2 ± 2.4	1.04 ± 0.45	50.50%	1668.3 ± 661.9	53.10%	7.0 ± 2.5[b]	45.5
Tumor injection × 4	10/10	16.0 ± 0.6/18.6 ± 1.3	1.12 ± 0.55	46.70%	1869.8 ± 846.6	47.40%	8.5 ± 4.4[b]	55.2
Tumor injection × 4	10/10	16.2 ± 0.8/18.5 ± 0.8	1.23 ± 0.39	41.40%	2200.3 ± 826.5	38.10%	10.1 ± 4.2[a]	65.6

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; *Z*: Inhibition rate of tumor weight; *J*: Inhibition rate of tumor volume; RTV: Relative tumor volume.

animals being similar in each group: control group, high dose group (1×10^7 pfu/tumor), middle dose group (5×10^6 pfu/tumor) and low dose group (5×10^5 pfu/tumor). The drug was administered via intratumoral injections of 0.1 mL/tumor on the first day.

Statistical analysis

Data were expressed as mean ± SD. The inhibition rate of tumor proliferation = (tumor weight of control group - tumor weight of drug group)/tumor weight of control group × 100%. Tumor volume (V) = $1/2ab^2$ (a = tumor major diameter; b = tumor minor diameter). The inhibition rate of tumor volume proliferation = (tumor volume of control group - tumor volume of drug group)/tumor volume of control group × 100%. Relative tumor volume (RTV) = Vt/Vo (Vo = tumor volume pre-drug, Vt = tumor volume measured each time after drug administration). The relative tumor proliferation rates (T/C) = RTV of drug group/RTV of control group × 100%. SPSS13 was used for statistical analysis of inter-group difference using t tests and for plotting of the tumor volume, relative growth curve of volume-time, tumor weight and related tables and figures.

RESULTS

The tumor volume on day 15 post-treatment in the control group was 3555.8 ± 1849.8 mm^3. The tumor volume of the group treated with a single intratumoral injection of low dose virus was 2200.3 ± 826.5 mm^3 ($P < 0.05$ *vs* control). For the middle dose virus group, the tumor volume on day 15 post-treatment was 1869.8 ± 846.6 mm^3 ($P < 0.05$ *vs* control). The tumor volume on day 15 post-treatment of the high dose virus group was 1668.3 ± 661.9 mm^3 ($P < 0.01$ *vs* control) (Figure 2A). The inhibition rates of the tumor volumes of dose of the low, middle, and high dose groups were 38.1%, 47.4% and 54.3%, respectively. Thus, $HSV^{GM\text{-}CSF}$ could inhibit pancreatic cancer in a dose-dependent manner. The relative tumor proliferation rates of the low, middle, and high dose groups were 45.5%, 55.2% and 65.5%, respectively (Table 1).

The present study showed that the tumor weight of the control group was 2.10 ± 1.41 g, 1.23 ± 0.39 g in the low dose group ($P > 0.05$ *vs* control), 1.12 ± 0.55 g in the middle dose group ($P > 0.05$ *vs* control), and 1.04 ± 0.45 g in the high dose group ($P < 0.05$ *vs* control). The inhibition rates of the tumor weights of the low, middle, and high dose groups were 41.4%, 46.7% and 50.5%, respectively (Figure 2B). Only the high dose group showed a significant difference compared with the control group ($P < 0.05$). There was no significant difference in mouse body weight among these four groups ($P > 0.05$) (Figure 3). Also, none of the mice died or showed skin ulceration/necrosis at the tumor location during the experiment.

The results of serum GM-CSF protein level showed that $HSV^{GM\text{-}CSF}$ significantly increased GM-CSF production, peaking at day 3 after treatment (Figure 4). There may be a correlation between the dose of $HSV^{GM\text{-}CSF}$ and the GM-CSF protein level.

Dissection of the mice at 15th day after administration of the drug showed no adhesions around the tumors, and there were no ascites or metastasis of the tumors in the peritoneal cavity; the tumors appeared as gray in color, had uniform textures and showed no necrosis.

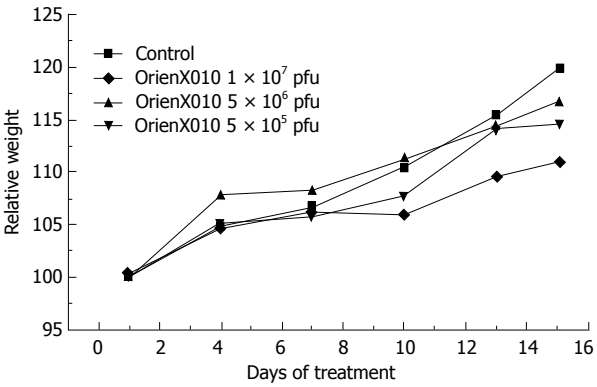

Figure 3 Changes in relative mouse body weights. pfu: Plaque forming units.

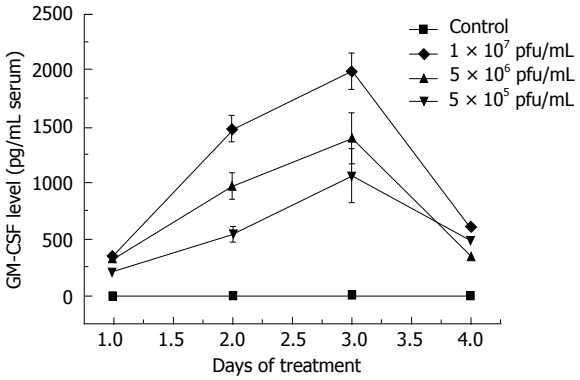

Figure 4 Quantification of expressed granulocyte-macrophage colony-stimulating factor. pfu: Plaque forming units; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

DISCUSSION

Compared to the traditional therapeutic methods, gene therapy is a recent and active research field. Since 1999, Germany, the United Kingdom and the United States have approved gene therapy projects for pancreatic carcinoma to enter clinical stage Ⅰ/Ⅱ trials, some of which are complete, providing preliminary data about its safety and effectiveness. The data suggested that the gene drugs were well tolerated in cancer patients and could suppress tumor growth[3-7]. The aim of the present study was to evaluate the efficacy of HSV$^{GM\text{-}CSF}$, an attenuated, replication-competent oncolytic virus, for treating mouse pancreatic carcinoma.

HSV$^{GM\text{-}CSF}$ is an engineered oncolytic virus. It belongs to a conditional replication HSV-1 mutant that uses the differences in cellular structure and metabolic pathways between tumors and normal tissues and retains the genes related to virus replication. The key features of HSV$^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* genes, as well as interruption of the *ICP6* gene and insertion of the therapeutic gene *GM-CSF*. GM-CSF is a pleiotropic cytokine secreted by many kinds of cells, including activated lymphocytes, macrophages and endothelial cells. Several previous studies demonstrated that GM-CSF was one of the most potent cytokines[5] that could influence the immune response in several ways, including recruitment and stimulation of antigen-presenting cells, such as dendritic cells, and induction of myeloid precursor cells to proliferate and differentiate into monocytes, macrophages, neutrophils and eosinophils[10]. Viral lysis and the mechanism mediated by the transgene protein, represent two parallel mechanisms of tumor destruction that can be achieved using HSV$^{GM\text{-}CSF}$.

The encouraging results of the present study suggested that the proliferation speed of tumors in the mouse experimental groups was reduced after 15 d of administration of HSV$^{GM\text{-}CSF}$ compared with the control group ($P < 0.05$). The reduction of tumor growth was dose-dependent. However, there was no obvious difference in the host response between the different dosages, which may be related to the small differences in drug dosages and could be resolved with the promotion of pharmacological techniques for high-concentration drugs.

The sera of cells infected with the three doses of the virus showed high expression of GM-CSF. There was no GM-CSF secretion in the control group. The results suggested that the HSV$^{GM\text{-}CSF}$ enhanced *GM-CSF* gene expression. Additionally, the increase was more pronounced in the group injected by the high dose virus than in the middle and low dose groups. There may be a correlation between the dose of HSV$^{GM\text{-}CSF}$ and the GM-CSF protein level. These results indicated that HSV$^{GM\text{-}CSF}$ could regulate immunity in cancer-bearing mice. The increased GM-CSF levels might be responsible for the dose-dependent relationship between the drug and the tumor response.

Currently, HSV vectors alone, and HSV vectors armed with GM-CSF or other recombinant genes have been successfully tested for safety in humans and have exhibited efficacy in preclinical animal models against various human cancers. And HSV mutant has been shown to be an effective strategy for lysing tumor cells *in vitro* and in multiple experimental animal models[10-16]. Geevarghese *et al*[17] evaluated the anti-tumor effects of NV1020 (another HSV-1 mutant), which showed that the NV1020 stabilized liver metastases in patients, and extended survival by resensitizing the cancer cells to chemotherapy. Both Yang *et al*[18] and Malhotra *et al*[19] suggested that the HSV vectors armed with GM-CSF had a significantly better antitumor effect compared to treatment with HSV vectors alone in mouse colon cancer. Furthermore, Derubertis *et al*[20] declared that mouse colorectal cancer hepatic metastases could be suppressed by HSV vectors armed with GM-CSF. HSV$^{GM\text{-}CSF}$ combined with cisplatin-based chemoradiotherapy was well tolerated in patients with stage Ⅲ/Ⅳ head and neck cancer. The present study showed that HSV$^{GM\text{-}CSF}$ enhanced the inhibition rate of mouse pancreatic cancer by regulating the expression of GM-CSF. The results were similar to those provided in previous studies[19,20].

Although the agent was highly attenuated and replication restricted, the use of a virus still raises concerns about viral proliferation and dissemination. During the experimental period, the body weights of the mice in the

experimental groups were similar to the control group at the beginning, and gradually and stably increased. At the later stages, the body weights slowly increased, particularly in the high-dosage group, compared to the control group, but there was no statistical difference. In addition, there was no occurrence of treatment-related death or ulceration/necrosis of the skin, suggesting that the drug is safe and effective, with low toxicity and side effects, and is tolerated by mice. The existence of antiviral drugs, such as ganciclovir, provides us with a further margin of safety.

In the present study, the transplanted tumor cell was injected into the armpits of mice and the drug was administrated by intratumoral injection. If applied in a clinic, the drug could be administered through a fine needle puncture technique with the guidance of CT/endoscopic ultrasonography or through vascular intervention, which several research centers have proved to be effective. Mulvihill *et al*[21] performed a clinical trial of intratumoral injection of the *ONYX-015* gene with the guidance of CT, while Löhr *et al*[22] reported their experimental results of clinical stage I and II trials of drug administration via vascular intervention.

Previous studies indicated that the HSV vector had a significant effect on multiple solid tumors[23-26], and could enhance the effect of other combined common therapies, such as radiotherapy and chemotherapy. Most studies that combined viral gene therapy with other therapies observed a synergistic effect in preclinical models[27-29]. Recently a stage I / II clinical trial of combined HSV with radiotherapy in head and neck tumors ended and showed no obvious side effects[7]. We are performing experimental research using an injection solution of recombined mouse $HSV^{GM\text{-}CSF}$ combined with radiotherapy to find a new approach in treating pancreatic carcinoma.

During the last two decades, gene therapy has made great progress. Simultaneous use of basic research and clinical experiments may become one of the fastest-developing areas in the field of medicine in the next 10 years. The development of gene therapy has proved difficult, and application in the clinic is still a long way off. The immunity, safety, transduction rate and tissue specificity of current vectors require further study and improvement, which is a common problem in gene therapy. The vectors used in the clinic in the future should have the advantage of combining non-viral vectors and alternative viral vectors that can be customized according to different requirements to express the target gene in specific tissues, and effectively modulate their expression level and duration.

COMMENTS

Background

Pancreatic cancer is a rapidly fatal malignancy with one-year relative survival rates less than 30%; nearly all patients die from their disease within 7 years of surgery. Gene therapy of pancreatic carcinoma is considered a novel model, and has become an emerging research area in recent years.

Research frontiers

The gene therapy model for pancreatic carcinoma has emerged recently. Successful drugs for gene therapy may result in prolonged survival. Oncolytic herpes simplex virus encoding granulocyte-macrophage colony-stimulating factor ($HSV^{GM\text{-}CSF}$) is an attenuated, replication-competent oncolytic virus. It can activate the host's own immune system against infected tumor cells. Some clinical trials of HSV for the treatment of various cancers had been completed, providing preliminary data about its safety and effectiveness. However, there is little data for pancreatic cancer.

Innovations and breakthroughs

$HSV^{GM\text{-}CSF}$ is an engineered oncolytic virus. It is a conditional replication HSV-1 mutant that utilizes differences in cellular structure and metabolic pathways between tumor and normal tissues, and retains the genes related with virus replication. The key features of $HSV^{GM\text{-}CSF}$ include the deletion of both copies of $\gamma_1$34.5 and *ICP47* gene as well as interruption of the *ICP6* gene and insertion of the therapeutic gene GM-CSF.

Peer review

The study is very interesting. Liu *et al* investigated the therapeutic efficacy of oncolytic $HSV^{GM\text{-}CSF}$ in a mouse model of pancreatic carcinoma and explored mechanisms that may be involved in the antitumor response. The authors provide evidence that $HSV^{GM\text{-}CSF}$ could inhibit the growth of pancreatic cancer.

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