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Intraperitoneal Delivery of hrR3 and Ganciclovir Prolongs Survival in Mice with Disseminated Pancreatic Cancer

HIDEKI KASUYA, MD,[1] YUKIHIRO NISHIYAMA, MD,[2] SHUJI NOMOTO, MD,[1] JIRO HOSONO, MD,[1] SHIN TAKEDA, MD,[1] AND AKIMASA NAKAO, MD[1]*

[1]*Department of Surgery II, Research Institute for Disease Mechanism and Control, University School of Medicine, Nagoya, Japan*

[2]*Laboratory of Virology Nagoya, Research Institute for Disease Mechanism and Control, University School of Medicine, Nagoya, Japan*

Background and Objectives: Intraperitoneal dissemination of pancreatic cancer is associated with a poor prognosis. Surgical resection does not prolong survival. Here we describe a novel approach to this difficult clinical problem consisting of intraperitoneal delivery of the herpes simplex virus (HSV) vector (hrR3) to mice with peritoneal dissemination of the pancreatic cancer cells.

Methods: The human pancreatic cancer cell line (SW1990) was implanted into the abdominal cavity of nude mice. Fifteen days later, the abdominal neoplasm was treated by intraperitoneal injection of the replication-conditional HSV vector (hrR3). The mutant lacks the ribonucleotide reductase gene, but contains an intact HSV-tk gene. Beginning 5 days after vector injection, mice were treated with a 14-day course of ganciclovir.

Results: Long-term survival (150 days) was seen in 70% of mice receiving hrR3 and ganciclovir, 40% of mice receiving hrR3 alone, and 0% of untreated mice. No vector-related mortality was observed. X-Gal tissue staining revealed blue-stained cells only in tumor nodules, not in normal organs.

Conclusions: Intraperitoneal delivery of hrR3 and ganciclovir improves survival in this murine model of peritoneal dissemination of pancreatic cancer. The ability of hrR3 to replicate only in rapidly dividing cells makes this virus an attractive vector for gene therapy of cancer.

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KEY WORDS: **disseminated pancreatic cancer; herpes vector; HrR3; ganciclovir; intraperitoneal delivery**

INTRODUCTION

Pancreatic cancer is one of the most difficult cancers to diagnose early and treat curatively [1,2]. Peritoneal dissemination is the most frequent type of recurrence after surgical resection [3,4], and this condition is generally refractory to chemotherapy, radiotherapy, and endocrine or immune therapies, often culminating in a fatal outcome [5]. Moreover, peritoneal dissemination of pancreatic cancer often is associated with intractable ascites, leading to substantial impairment of the patient's quality of life. Thus, the development of a new modality of treatment for this condition has been eagerly awaited [6]. Gene therapy for intraperitoneal spread of pancreatic cancer may confer a more favorable risk-benefit ratio than conventional treatments.

We have investigated a novel therapeutic strategy us-

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*Correspondence to: Akimasa Nakao, M.D., Department of Surgery II, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.

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ing the vector hrR3 based on the herpes simplex virus type 1 thymidine kinase (HSV-tk)/ganciclovir (GCV) paradigm in a mouse abdominal tumor model. hrR3 lacks the ribonucleotide reductase (RR) gene, which is a key enzyme in the biosynthesis of DNA in all prokaryotic and eukaryotic cells. This deficiency renders the mutant virus replication-competent only in dividing cells. Therefore, replication of the virus is restricted largely to tumors, and normal organs are protected from the adverse effect of treatment. hrR3 possesses an intact HSV-tk gene that can be used for metabolic activation of GCV, which acts to disrupt cellular and viral DNA replication. The phenomenon that HSV-tk–expressing cells also can induce cell death in neighboring cells, which do not express HSV-tk, has been called the bystander effect, and it may be due to induction of apoptosis [7]. We hypothesized that the combination of hrR3 and GCV would improve outcome when used to treat intraperitoneal dissemination of pancreatic cancer. In this study, we thus investigated its effect in a mouse model.

MATERIALS AND METHODS

Cell Lines

The SW1990 cell line, derived from human pancreatic cancer, was provided by Dr. T. Sawada (First Department of Surgery, Osaka City University, Osaka, Japan) [8]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin (Sigma, Tokyo, Japan). We have previously described the dissemination of SW1990 in the abdominal cavity after intraperitoneal inoculation [9].

Vectors

hrR3, an RR (ICP6)-deficient HSV mutant, was kindly provided by Sandra K. Weller (University of Connecticut Health Center, Farmington, CT) [10].

Animal Studies

Animal studies were performed in accordance with guidelines issued by the Nagoya University animal center (6-week-old female BALB/c-nu/nu) were obtained from Charles River Japan Co., Yokohama, Japan. Mice were anesthetized using diethyl ether, the peritoneal cavity was opened, and the pancreas was identified. Typically, intraperitoneal inoculation of 1×10^7 SW1990 cells into BALB/c nude mice near the pancreas resulted in the formation of multiple white nodules <0.5 mm in diameter on the mesentery around the pancreas within 1 week. The condition of the animals was checked once or twice a day for the duration of the study. During this observation period, euthanasia of terminally ill animals was carried out using an overdose of pentobarbital. The peritoneal cavities of euthanized mice were examined macroscopically for terminal tumor disease. Mice were divided randomly into 4 groups. Mice in groups A (n = 10) and B (n = 10) were inoculated with replication-conditional 1×10^8 infectious particles of hrR3 in 1 ml of DMEM on day 15 after neoplasm implantation. Mice in group C (n = 10) received 1 ml of DMEM intraperitoneally (ip) on day 15 after neoplasm implanted. Group A received GCV (0.4 mg ip, twice a day) starting on day 5 after vector injection and continuing for 14 days. Control groups B and C received two daily intraperitoneal injections of 0.9% NaCl during this period. Mice in group D (n = 10) were not inoculated with SW1990 cells but received replication-conditional 1×10^8 infectious particles of hrR3 in 1 ml of DMEM ip, followed by GCV treatment as above.

Histopathological and Histochemical Studies

Survivors from each group (A, 7; B, 4; C, 0) were killed by an overdose of pentobarbital and autopsied on day 150 after neoplasm inoculation. One mouse from group B, which died on day 10 after vector inoculation, also was autopsied within 12 h of death. Tissues were harvested and rapidly frozen in liquid nitrogen. Cryostat sectioning of the tissue was performed at 10 μm thickness with a microtome. The sections were then fixed and stained with X-Gal.

RESULTS

Long-term survival (LTS: 150 days) was achieved in 70% of mice treated with intraperitoneal injection of hrR3 followed by systemic GCV treatment (Fig. 1A). Mice that had received intraperitoneal injection of the vector alone (Fig. 1B) showed 40% LTS. Untreated mice (C) showed 0% LTS. Mice that were not inoculated with SW1990 cells but received hrR3 followed by GCV showed 100% LTS. Statistically significant differences existed between groups A and C ($P = 0.0014$), and between groups B and C ($P = 0.0020$) using the log-rank test. However, there was no statistically significant difference between groups A and B ($P = 0.283$).

None of the mice in groups receiving hrR3 displayed neurologic symptoms or other toxic side effects after vector injection.

To assess the spread of virus in the abdominal cavity, histopathological and histochemical examination was performed in mice of group B on days 11, 28, 57, 72, and 136 following vector inoculation. In all of the mice, a number of neoplastic nodules were observed throughout the abdominal cavity with Douglas pouch peritoneal dissemination (Fig. 2), but the size of the tumors in vector-treated mice was significantly smaller than that in mock-treated mice. *LacZ* histochemistry revealed many blue cells in the neoplastic nodules in vector-treated mice (Fig. 3), and virus-induced cell damage and inclusion bodies were observed in the region containing blue-stained cells. Tumors displayed inflammatory infiltrates

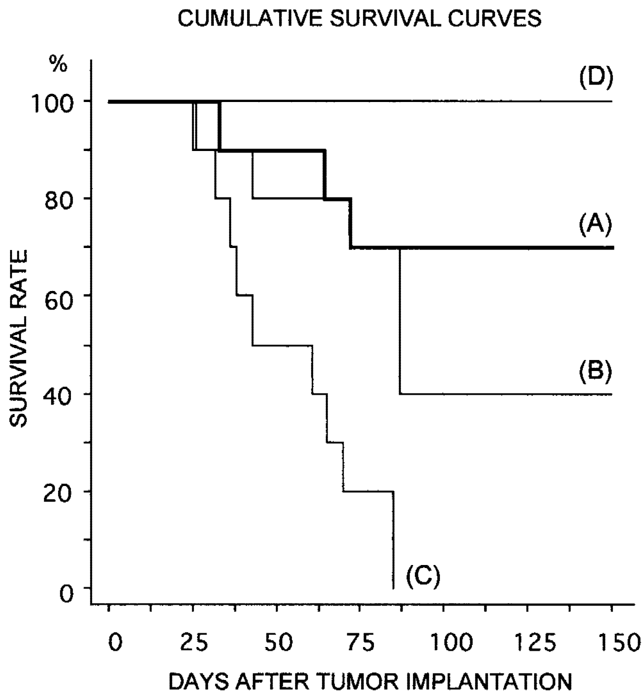

Fig. 1. Long-term survival (150 days after implantation) after hrR3/GCV treatment in a mouse peritoneal dissemination model using the SW1990 pancreatic cancer cell line. Fifteen days after implantation, abdominal tumors were treated by intraperitoneal (ip) injection of the replication-conditional HSV vector hrR3. Five days after vector injection, ip injection of ganciclovir (GCV) or saline was carried out for 14 days: group A, SW1990 + hrR3 + GCV; group B, SW1990 + hrR3 + saline; group C, SW1990 + saline; group D, hrR3 + GCV. Statistical differences in survival were determined by log-rank analysis (group A vs. B, $P = 0.283$; group A vs. C, $P = 0.0014$; group B vs. C, $P = 0.0020$).

with a few neutrophils and minimal lymphocytes, and also revealed degenerative and necrotic change (Fig. 4). Neither blue cells nor significant cell damage was observed in normal tissues of the liver, spleen, pancreas, gastrointestinal tract, ovary, or mesenteric membrane in group B. LTS mice (150 days) in group B (4) had abdominal tumors and mucinous ascites; one of the survivors had *LacZ* staining indicating the presence of the HSV vector in some of the tumor cells.

DISCUSSION

Metastasis from pancreas cancer is usually fatal. Gene therapy using a combination of the hrR3 vector and GCV represents a novel approach for treating peritoneal metastasis from pancreatic cancer. In the present study, we found that intraperitoneal delivery of HSV-tk–positive, replication-conditional HSV vector combined with GCV treatment yielded LTS in 70% of mice with SW1990 pancreatic tumors in the peritoneal cavity. The histopathological examination demonstrated that the characteristic blue color of the X-Gal reaction was observed only in neoplastic cells, but not in the peritoneal membrane or normal organs, in the peritoneal cavity.

The distribution of hrR3 in the Douglas pouch further demonstrated that the vector reached disseminated tumors on day 10 after inoculation into the peritoneal cavity. The widespread distribution of the vector most likely is due partly to the ability of replication-conditional vectors, including hrR3, to propagate in mitotic neoplastic cells, thereby increasing the number of infectious vector particles in neoplastic tissue. Such ability to achieve disseminated gene transfer undoubtedly contributes to the therapeutic outcome. Retroviral and adenoviral vectors usually are replication-defective and do not produce new vector particles after infection of tumor neoplastic cells. This may be why intratumoral or intracavitary injection of herpes vectors transduces tumor cells in vivo more efficiently than adenovirus or retroviral vectors [11,12]. In addition, since the hrR3 vector lacks the RR gene, DNA replication of hrR3 is compromised in nondividing cells but not in highly mitotic cells such as tumor cells [10].

There is another advantage to the hrR3 vector. Unlike adenovirus and retroviral vectors, hrR3 can be effectively controlled by GCV because it contains an intact HSV-tk gene. In fact, hrR3 is hypersensitive to GCV, as we described in a previous study [13]. The high transduction efficiency of HSV compared with other viral vectors also may have contributed to the success of this study.

Retroviral vector gene therapy followed by GCV in the treatment of disseminated pancreatic cancer has been reported: The retroviral vector survives in the peritoneal cavity, and the efficacy of retroviral HS-tk/GCV treatment of cultured human pancreatic cancer cells exposed to ascites fluid has been demonstrated [14]. Hwang et al. [15] also describes gene therapy using the retroviral vector bearing wild-type *P53* in the treatment of disseminated pancreas cancer. Treatment of nude mice with the retroviral *P53* vector results in a significant inhibition of the primary pancreatic neoplasm, as well as peritoneal neoplasm deposits. Gene therapy for peritoneal dissemination of pancreatic cancer by liposome-mediated transfer of the HSV-tk gene has demonstrated HSV-tk gene expression in neoplastic cells but not in normal pancreas or in the small intestine [16].

Despite these promising findings, survival data were not shown in those papers. One major limitation of the HSV vector–based approach may be the small therapeutic window required to prevent toxic side effects, especially in treatment of central nervous system neoplasms [17]. In the present study, using the hrR3 vector, mice did not display any neurologic findings or side effects. Intraperitoneal virus injection appears safer than intrathecal injection for treating brain disease. The HSV-tk/GCV paradigm not only functions as an antineoplastic agent but also can decrease HSV vector–related toxicity by blocking HSV replication. There was some difference in the survival rate between groups A and B, although it

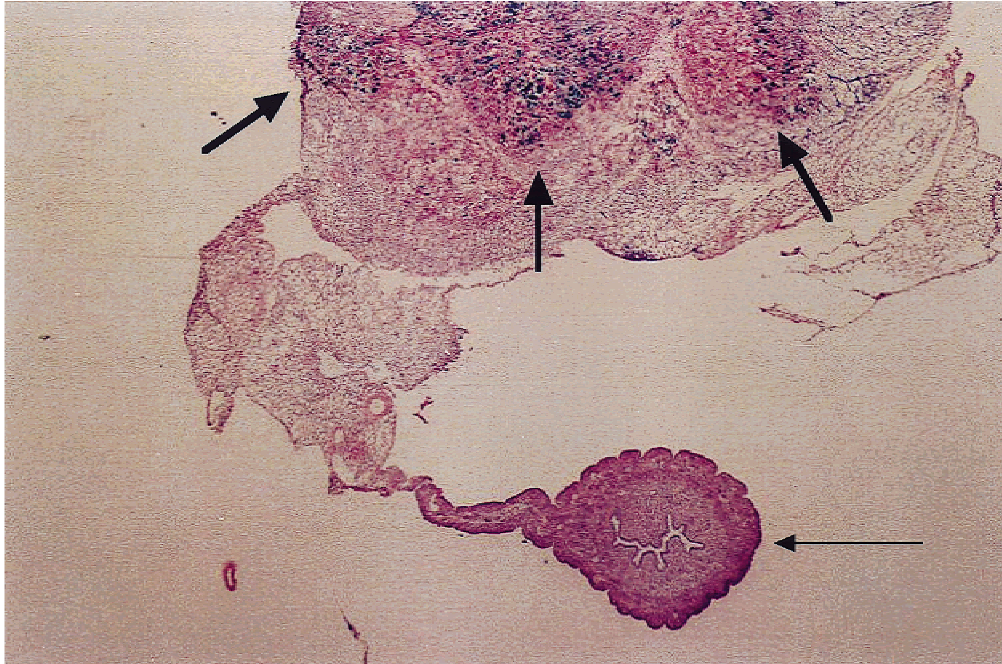

Fig. 2. Distribution of blue staining, indicating the presence of hrR3 in the peritoneal cavity (×20). Douglas pouch peritoneal dissemination of pancreatic neoplasm was found in a mouse from group B that died 25 days after implantation. The blue cells were detected in neoplastic tissues on the peritoneal membrane. No blue cells were detected in the retroperitoneal membrane, intraperitoneal fat, or other organs (large arrows, blue cells in metastatic pancreas cancer of ovary; small arrow, ovarian duct).

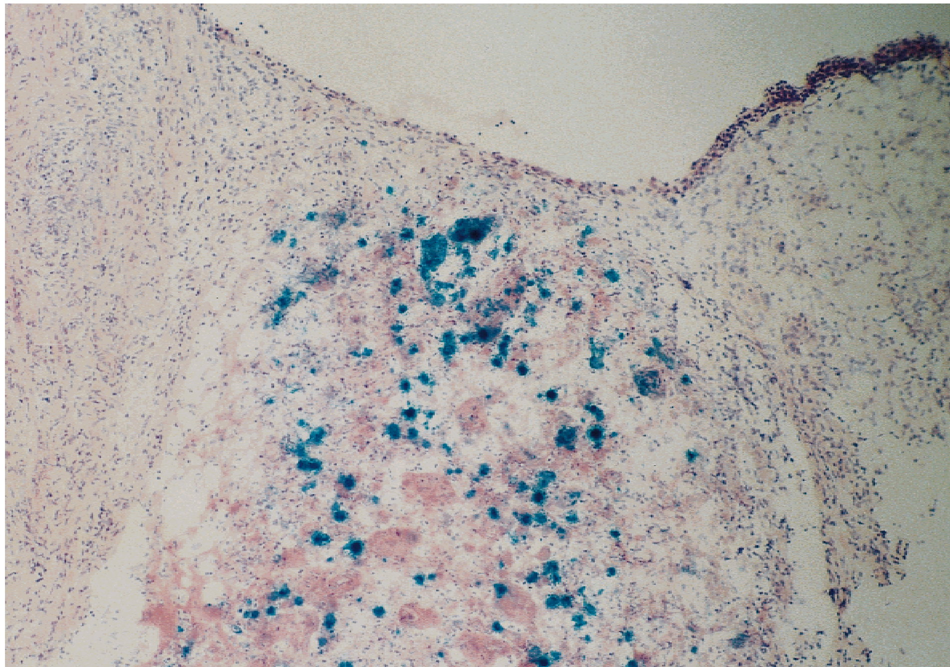

Fig. 3. Inflammation and cytocidal effect were found in the blue-staining mass from a mouse in group B that died 25 days after implantation (×100).

was not statistically significant. It is possible that the bystander effect may be involved in the higher survival rate of group A.

Non-specific immune response to the virus-infected cells may facilitate an antineoplastic immune response. Only in the tumors could we find many clear nuclei with viral inclusion bodies. Inflammation was found around and in the tumors, with some neutrophil infiltration and

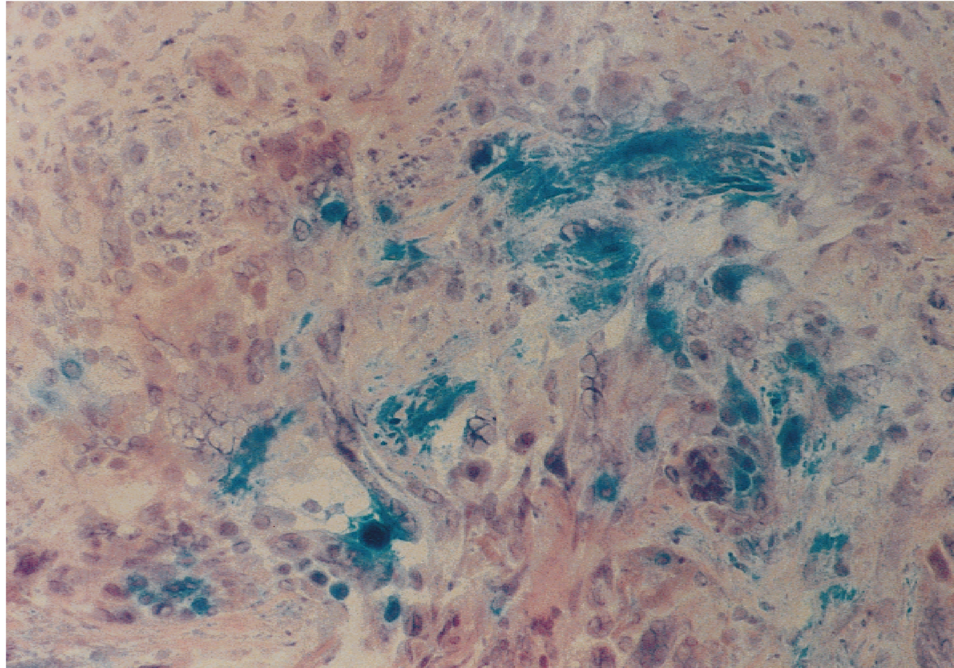

Fig. 4. Only the tumors had many clear nuclei containing viral inclusion bodies. Thin, compressed chromatin was found in these clear nuclei. Neoplastic tissue revealed degenerative and necrotic change (×200).

minimal lymphocytes. The immune response may also increase safety by limiting spread of the vector in the peritoneal cavity.

About 90% of humans have circulating antibodies to HSV, and most of them harbor HSV wild-type virus in latency. The possibility that these latent wild-type viruses might be reactivated by application of HSV vectors cannot be excluded, although both virus and vector replication could be blocked by GCV. The outcome of the therapeutic strategy presented here should encourage further development of safer HSV vectors, including new replication conditions that reduce toxicity. We describe the high therapeutic potential and the possible control of HSV toxicity. These data support the further testing of such new HSV vectors in nonhuman primates with a view to possible clinical trials in disseminated pancreatic cancer.

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