Oncolytic Viral Therapy for Cervical and Ovarian Cancer Cells by Sindbis Virus AR339 Strain

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Abstract

Purpose: Recently, the application of replication-competent viruses has been studied as anticancer agents. Sindbis virus (SIN) is an RNA virus that belongs to the Alphavirus genus in the Togaviridae virus family. The AR339 strain of SIN has not been reported to induce any serious disease to humans.

Experimental Design: In this study, we evaluated the feasibility of the replication-competent SIN AR339 strain as an agent for cervical and ovarian cancer therapy.

Results: SIN infection was able to induce cytopathic effects and apoptosis in two cervical cancer cells (HeLaS3 and C33A) and three ovarian cancer cells (HOC-1, HAC-2, and OMC-3) but not in normal human keratinocytes in vitro. The analysis of cell viability, virus protein synthesis, and viral growth showed the cancer-specific cytoxicity and virus growth of SIN. In nude mice, i.t. and i.v. inoculation of SIN resulted in significant regression of established cervical tumors implanted at their backs.Histologic studies revealed that systemic treatment with the single injection of SIN induces necrosis within tumors at a remote site. In the metastasis model of ovarian cancer, suppression of ascites formation was observed in nude mice with i.p. SIN treatment. By using an in vivo green fluorescent protein imaging system, we also showed that systemic treatment with SIN targeted tumors specifically.

Conclusions: Our study suggested that SIN AR339 strain has a possibility as a novel agent for human cervical and ovarian cancer therapy.

The limited efficacy and the toxicity of conventional cancer therapies have prompted the search for novel approaches. One such approach is cancer gene therapy, for which replication-defective viruses are employed as vectors. Several viral vector systems are used for introduction of genes, such as those encoding suicide proteins, tumor suppressor proteins, or cytokines, into tumor cells. However, the effect of this approach is limited because these vectors transduce these genes into only a small portion of the target cells. Therefore, replication-competent viruses have been studied as anticancer agents (1–3). Sindbis virus (SIN) is an RNA virus that belongs to the Alphavirus genus in the Togaviridae virus family, and is transmitted to birds and mammals by mosquito bites (4) and subsequently spreads throughout the body via the bloodstream (5). SIN has the potential to induce apoptosis in infected mammalian cells (6, 7).

In addition, one of the surface receptors on mammalian cells to mediate SIN infection has been revealed as the 67 kDa high-affinity laminin receptor (8), which has been reported to be highly expressed in various human cancers compared with normal cells (9, 10). In humans, SIN infection is considered to induce no symptoms or only mild symptoms (fever, rash, and arthralgia; ref. 4). Therefore, several replication-defective SIN vector systems have been studied for in vitro gene transfer to mammalian cells and in vivo gene therapy (5, 11). However, it has not been examined particularly whether replication-competent wild-type SIN can be used as an anticancer agent.

In this study, we tested normal human keratinocytes, two human cervical, and three ovarian cancer cell lines for their susceptibility to SIN infection in vitro, compared with the infection of reovirus, which is well known as oncolytic virus to target cancer cells with an activated Ras pathway (12). We also evaluated the ability of SIN to suppress the progression of cervical and ovarian cancer cell tumors implanted in immune-compromised mice. Finally, using an in vivo green fluorescent protein (GFP) imaging system, we examined whether SIN has the potential for systemic delivery and tumor-specific infection. Our study indicated that replication-competent SIN could be a practical novel strategy against cervical and ovarian cancers in the future.

Materials and Methods

Cell culture. Human cervical cancer cell lines (HeLaS3 and C33A) and BHK-21 cells were obtained from the American Type Culture
Collection (ATCC; Rockville, MD). The cells were maintained according to ATCC protocols. Vero cells were laboratory stock and were maintained in DMEM supplemented with 10% FCS at 37°C in 5% CO₂. Human ovarian cancer cell lines (HOC-1, HAC-2, and OMC-3) were kindly provided as follows: HOC-1 from Dr. T. Fujii (Hamamatsu University School of Medicine, Japan); HAC-2 from Dr. M. Nishida (Tsukuba University, Japan); and OMC-3 from Dr. T. Yamada (Osaka Medical College, Japan; ref. 13). HOC-1 and HAC-2 cells were maintained in RPMI 1640 supplemented with 10% FCS and OMC-3 cells were maintained in DMEM supplemented with 10% FCS at 37°C in 5% CO₂.

As previously described, we prepared primary cultured cells from normal cervical tissues, which were obtained from surgical specimens confirmed by histologic examination to be normal (14).

Viruses. SIN AR339 (wild-type) used in this study was previously provided by the National Institute of Infectious Diseases (Tokyo, Japan). The virus was propagated in primary chicken embryo fibroblast cells and then passaged several times on Vero cells. This virus was used as laboratory stock. The GFP-expressing recombinant SIN (TR339-GFP/2A) was derived from cDNA clone pTR339-GFP/2A, which was kindly provided by Dr. Hans W. Heidner (University of Texas, San Antonio, TX; ref. 15). The cDNA clone was linearized by digestion with XhoI, and a run-off transcript was produced by using SP6 RNA polymerase. The RNA transcript was then electroporated into C33A cells, and a virus-containing growth medium was collected at 48 hours after electroporation and frozen at −80°C. AR339 and TR339-GFP/2A were propagated in C33A cells maintained in DMEM supplemented with 10% FCS at 37°C in 5% CO₂, and viral titers were determined by plaque assays using monolayers of the BHK-21 cells.

The reovirus strain T3D used in this study is a laboratory stock. The virus was propagated in L cells (from ATCC) maintained in

![Fig. 1. A, cytopathic effects of SIN and reovirus infection. Normal human keratinocytes (a, g, and m), cervical (b, c, h, i, n, and o), and ovarian (d, e, j, k, l, p, q, and r) cancer cells were infected with mock (a, b, c, d, e, and f), SIN (g, h, i, j, k, and l), or reovirus (m, n, o, p, q, and r) at an MOI of 0.01. Cells were photographed 96 hours after virus infection. B, cell viability assays of cells infected with SIN (solid columns) or reovirus (hatched columns). Cells, 1 × 10⁴, were plated and infected with mock, SIN, or reovirus at indicated MOI for 96 hours. Keratinocytes (a), HeLaS3 (b), C33A (c), HOC-1 (d), HAC-2 (e), and OMC-3 (f) were stained with crystal violet in 25% (v/v) methanol. The surviving cells were measured by the absorbance at 450 nm with a plate reader. Cell viability of mock injection was calculated as 100%.](image-url)
DMEM supplemented with 10% FCS at 37°C in 5% CO₂, and viral titers were determined by plaque assays using monolayers of the L cells.

Cytotoxicity in cell lines. Cells were plated onto 24-well plates at 1 x 10⁴/well and infected with SIN or reovirus at a multiplicity of infection (MOI) of 0.01, 0.1, or 1. After a 96-hour incubation, the medium was removed, and cells were stained with crystal violet in 25% (v/v) methanol for 30 minutes, and then gently rinsed with water. Cell viability was evaluated by the absorbance measured at 450 nm with a plate reader (Wallac 1420 ARVOsx Multilabel Counter, Perkin-Elmer, Chiba, Japan). Cell viability of mock injection was calculated as 100%. These data were presented as mean and SD of three determinations.

Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay. The 5.0 x 10⁴ cells seeded on Lab-Tek chamber slides (Nalge Nunc International) were then incubated in the presence or absence of SIN at an MOI of 1 for 24 hours. The cells on chamber slides were then washed twice with PBS, air dried, and fixed with 4% paraformaldehyde at room temperature for 30 minutes. The terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done by using an In situ Apoptosis Detection Kit (TaKaRa, Tokyo, Japan) according to the instructions of the manufacturer. Cells were viewed and photographed under a fluorescence microscope (Nikon, Inc., Tokyo, Japan).

Virus growth in cell lines. The cell lines grown in 24-well plates were infected with SIN or reovirus at an MOI of 0.1. After a 96-hour...
incubation, culturing medium was collected. After freezing and thawing once, viral titers were determined by plaque assays using BHK-21 cells or L cells.

**Preparation of anti–Sindbis virus antibody.** The SIN was emulsified with Freund’s complete adjuvant and injected s.c. at one site in the back of the rabbit. Immunization was carried out four times at 2-week intervals. Two weeks after the first immunization, blood was obtained, and then sampled every 2 weeks. The serum was separated and stored at −80°C until required.

**Protein extraction and Western blot analysis.** Cells, cultured in 10-cm dishes, were infected with SIN at an MOI of 1. After 0, 12, 24, and 48 hours of incubation, the medium was removed, and cells were washed with PBS twice and collected. The cells were lysed in a whole-cell extract buffer containing 20 mM/L HEPES, 75 mM/L NaCl, 2.5 mM/L MgCl2, 0.1 mM/L EDTA, 0.05% Triton X-100, 0.5 mM/L DTT, 0.1 mM/L NaVO4, 2 μg leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay Kit) and stored at −80°C until use. Sample proteins were mixed with SDS-PAGE sample buffer and boiled for 5 minutes, and then equal amounts (100 μg) were run on a 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The blots were developed with an enhanced chemiluminescent ECL Plus detection kit (Amersham) according to the instruction of the manufacturer. Detection was done by using Fluor-S Multimager (Bio-Rad).

**Animal studies.** All animal experiments were done according to the Chiba University laboratory animal center guidelines. Nude mice (BALB/cAnNcrj-nu/nu, female, 6 weeks old) were obtained from Charles River Japan, Inc. For intraneoplastic treatment with SIN, 1 × 106 HeLaS3 or C33A cells in 100 μL of PBS were injected s.c. in the back of nude mice. When the diameter of the tumor derived from HeLaS3 or C33A cells reached 7 mm, the mice were randomly assigned to a control group and a SIN treatment group. In treatment group, SIN was injected i.t. at a dose of 1.0 × 106 plaque-forming units (pfu) in 100 μL of DMEM at days 0, 5, 10, and 15. In control mice, 100 μL of DMEM were injected i.t. For systemic treatment with SIN, nude mice bearing established C33A tumors in their backs were injected i.v. into the jugular vein with 100 μL of DMEM either containing 1 × 106 pfu of SIN or not. The volume of the tumors was measured every 3 days with calipers for 50 days, using the following formula: length (mm) × width (mm) × height (mm).

For the peritoneal dissemination model of human ovarian cancer, nude mice were injected i.p. with 2 × 106 OMC-3 cells in 100 μL of PBS. Seven days after inoculation with OMC-3 cells (day 0), two groups of six animals were treated i.p. with 2 × 106 pfu of SIN or 100 μL of DMEM at days 0, 7, and 14. The body weight of mice was measured every week. For this model, the increase in body weights of SIN-treated and DMEM-treated mice was compared with that of untreated healthy mice bearing no tumors.

**Histologic studies.** For histologic analysis, the tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Sections were subjected to H&E staining.

In vivo green fluorescent protein imaging. Mice bearing C33A tumors were injected with 1 × 106 pfu of GFP-SIN in 100 μL of DMEM into the jugular vein. GFP expression in tumor was examined every 12 hours after the infection by using an Olympus fluorescence stereo microscope (model SZX7) equipped with a reflected light fluorescence unit for GFP (SZX-RFL2-GFP), which was coupled with a Hamamatsu ORCA-ER cooled CCD camera.

**Results**

**Cytopathic effects of Sindbis virus compared to reovirus.** To determine the susceptibility of human cervical and ovarian cancer cell lines to SIN, cervical cell lines (HeLaS3 and C33A) and ovarian cancer cell lines (HOC-1, HAC-2, and OMC-3), as well as a primary cultured keratinocytes derived from normal cervical tissues, were infected with SIN at an MOI of 0.01, 0.1, or 1. For comparison, cells were also infected with reovirus at the same MOI. Ninety-six hours after SIN infection at an MOI of 0.01, strong cytopathic effects were observed in all of the cervical and ovarian cancer cell lines (Fig. 1A, h-l), and no morphologic changes were observed in normal keratinocytes (Fig. 1A, g) compared with control cells without virus infection (Fig. 1A, a-f). However, 96 hours after reovirus infection at an MOI of 0.01, only weak or very low cytopathic effects were observed in the cervical and ovarian cancer cell lines (Fig. 1A, n-r), as well as in normal keratinocytes (Fig. 1A, m).

In addition, cell viability assays indicated that SIN exhibited strong cytopathic effects in cervical and cancer cell lines at an extremely low MOI (0.01 MOI; Fig. 1B, b-f) but not in normal keratinocytes at even higher titers (0.1 and 1 MOI; Fig. 1B, a).

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On the contrary, reovirus failed to exhibit remarkable cytopathic effects in these cancer cells at a low MOI (0.01 MOI), and killed not only cancer cells but also normal keratinocytes at a relatively high MOI (1 MOI).

**Sindbis virus–induced apoptosis in cancer cell lines.** Viral infection often leads to an apoptotic response in infected cells. SIN also induces apoptosis in mammalian cells (6, 7). To determine whether infection with SIN induces apoptosis in keratinocytes, HeLaS3, and C33A cells, TUNEL assays were done (Fig. 2). TUNEL-positive cells were observed in HeLaS3 and C33A cells 24 hours after SIN infection at an MOI of 1 (Fig. 2, H and I), whereas very few positive cells were observed in cells without SIN infection (Fig. 2, C, G, and K) and in keratinocytes 24 hours after SIN infection (Fig. 2, D). TUNEL assays were also done with ovarian cancer cells. Similar results were obtained with the ovarian cancer cells (data not shown). It was confirmed that SIN induces apoptosis in cervical and ovarian cancer cell lines but not in normal keratinocytes.

**Virus growth and virus protein synthesis in cancer cells with Sindbis virus infection.** To examine whether the degree of cell...
cytotoxicity correlates with virus growth, virus titers were measured in the cancer cell lines 96 hours after SIN or reovirus infection (0.1 MOI). SIN growth was not observed in normal keratinocytes, whereas high growth of SIN was observed in cervical and ovarian cancer cells as well as in Vero cells (Fig. 3A). On the contrary, reovirus grew to lower titers in cervical and ovarian cancer cells, as well as in normal keratinocytes, than they did in L cells (Fig. 3B).

To further determine whether the cell cytotoxicity was induced by SIN replication, the SIN protein synthesis in cells was analyzed at 0, 12, 24, and 48 hours after SIN infection at an MOI of 1. Sample proteins were subjected to Western blotting with anti-SIN antibody as described in Materials and Methods. Viral protein synthesis in cervical cancer cells (HeLaS3 and C33A) was remarkable 24 hours after SIN infection, and in Vero cells 48 hours after SIN infection. However, viral protein synthesis was not detected in normal keratinocytes until at least 48 hours after SIN infection at an MOI of 1 (Fig. 3C). These findings were consistent with the data of virus growth.

In vivo effects of Sindbis virus on human cervical tumors. To assess the in vivo antitumor potential of the SIN, we evaluated the therapeutic effects of i.t. SIN injection on s.c. cervical tumor xenografts. Nude mice bearing HeLaS3 or C33A tumors in their backs received i.t. injections of SIN (1.0 × 10^6 pfu) or 100 μL of DMEM at days 0, 5, 10, and 15. Tumor volume was followed for 50 days. The mean tumor volumes in both HeLaS3 and C33A cervical tumors were found to be significantly reduced in mice treated i.t. with SIN compared with those of mock-treated mice (Fig. 4A and B). In addition, all mice in which tumors had disappeared after i.t. treatment with SIN have been in very good health and living more than 6 months to date (data not shown).

To further confirm the ability of SIN for the i.v. treatment of cervical cancer cell tumors at remote sites, we injected SIN into the jugular veins of nude mice bearing C33A tumors in their backs. Four times (at days 0, 5, 10, and 15) i.v. injections of SIN (1.0 × 10^6 pfu) resulted in the remarkable regression of tumor growth for 50 days (Fig. 4B). Histopathologic studies were done with C33A tumors treated with a single i.v. injection of SIN (1.0 × 10^6 pfu). H&E staining of tumor sections indicated that a much greater proportion of necrotic areas was occupied in SIN-treated tumors than in untreated control tumors (Fig. 4C). Furthermore, it was evident that viable tumors in untreated tumors and necrotic tissues in SIN-treated tumors could be observed at a higher magnification (Fig. 4D). Therefore, at least in immune-compromised mice, it is confirmed that systemic treatment of SIN was effective in tumor growth suppression at a remote site.

Effects of Sindbis virus in the peritoneal dissemination model of human ovarian cancer. Generally, in advanced ovarian cancers, the tumor spreads throughout the peritoneal cavity and induces the production of ascites. To examine the therapeutic effects of SIN on advanced ovarian cancers, nude mice were injected i.p. with 2 × 10^6 OMC-3 cells 7 days before SIN i.p. treatment (day 0). Mice were treated with i.p. injections of 2 × 10^6 pfu of SIN or 100 μL of DMEM (mock-treated control) at days 0, 7, and 14. No abdominal swelling was observed in SIN-treated mice at day 42, whereas abdominal swelling was obvious in mock-treated mice (Fig. 5A). Because of the ascites formation, body weight of mock-treated mice significantly increased compared with that of mice without tumor inoculation. In contrast, little increase in body weight was observed in SIN-treated mice (Fig. 5B). Peritoneal dissemination was observed in mock-treated mice at day 42 (Fig. 5C). H&E staining of tumor sections indicated that viable tumors could be observed in mock-treated mice (Fig. 5D).

Tumor-specific detection by GFP-SIN i.v. injection. To confirm the potential of the SIN for systemic delivery and tumor-specific infection, 10 mice bearing C33A tumors received single
injections of GFP-expressing recombinant SIN (GFP-SIN) into the jugular veins. Eight representative mice at 3 days postinfection were shown in Fig. 6. In all of the treated mice \((n = 10)\), tumor-specific GFP expression could be observed by 2 days after the GFP-SIN injection, and persisted for more than 10 days (data not shown). In addition, we examined the GFP counts in lysates from various organs (tumor, brain, heart, lung, liver, kidney, and intestine) of nude mice who received single injections of GFP-SIN into the jugular veins. It was confirmed that the GFP counts in tumor tissues were significantly high but not those in other organs 72 hours after the infections (data not shown).

**Discussion**

SIN has been known to show cytopathic effects on various established mammalian cell lines (16–18). In humans, it has been reported that SIN infection induces only mild symptoms (fever, rash, and arthralgia; ref. 4), which suggests low infectivity and viral replication in normal tissues.

To use replication-competent viruses for anticancer agents, it must be confirmed that a virus shows specific infection for the cancer cells and low cytotoxicity in normal cells. In this study, we showed that tested human cervical and ovarian cancer cell lines were susceptible to SIN infection, whereas normal cervical keratinocytes were resistant. Also, it was found that SIN induces cytopathic effects and exhibits high virus growth in primary cultured human fibroblasts and mouse fibroblasts *in vitro*; however, the evident virulence of SIN has not been observed in mice treated with multiple injections of SIN, and mice in which tumors had disappeared have been in healthy appearance more than 6 months to date (data not shown).

SIN exhibited strong cytotoxicity to cervical and ovarian cancer cells at a low MOI, whereas reovirus did only at a relatively high MOI. Virus growth assays with cervical and ovarian cancer cells showed that SIN grows to a higher titer than reovirus in these cancer cells. In addition, within 48 hours postinfection, SIN virus protein synthesis could be observed in cervical cancer cells, but not in normal keratinocytes. These findings suggested that the degree of cell cytotoxicity induced by these viruses correlates with that of their replication in the test cells. Therefore, it was likely that the cytotoxicity of replication-competent SIN to cancer cells was limited compared with replication-competent SIN.

The potential of systemic tumor targeting is an important factor to be used in oncolytic virus therapy. For example, retroviruses are inactivated by human serum (19, 20) and adenoviruses are unstable in the blood stream (21, 22), which
prevents their systemic delivery to tumors. By contrast, SIN is stable in the blood stream (23, 24), which is important for systemic treatment to target remote tumors. In this study, regression of cervical tumor xenografts was observed in SIN i.v. treated mice as well as in SIN i.t. treated mice. Histopathologic studies also showed that SIN induces extensive necrosis in C33A tumors. These findings revealed that active virus replication can occur in tumors remote from the administration site. Also, it was confirmed that the virus inactivated by UV exposure for 30 minutes did not exhibit antitumor effects in an in vivo model (data not shown).

We used replication-competent GFP-expressing recombinant SIN, which allowed tumor detection and in vivo imaging by the systemic administration at a lower titer than the replication-incompetent SIN vectors containing the firefly luciferase gene (25, 26). Tumor-specific GFP expression could be observed by 2 days after a single GFP-SIN injection, and persisted for more than 10 days (data not shown), indicating that SIN has a potential for tumor-specific infection. Accordingly, in vivo identification of cancer (cells or tissues) could be realized with administration of SIN to an animal by detection of a change in the fluorescence intensity, expression of a SIN protein, or the expression of a reporter gene when the recombinant SIN containing the reporter gene insertion was employed.

It has been reported that SIN may target tumor cells via the high molecular weight laminin receptor (26). However, we confirmed that this receptor is highly expressed in normal keratinocytes (data not shown). Therefore, the expression level of the receptor could not account for the difference in the susceptibility to SIN between normal keratinocytes and keratinocyte-derived cancer cells.

In conclusion, SIN therapy is effective in vitro and in vivo for all of cervical and ovarian cancers tested in this study. Currently, the main method of treatment for cervical and ovarian cancers is surgical resection, which is often followed by combination chemotherapy and radiotherapy in patients with advanced cancers. However, additional options have been required for a superior result of cancer therapy. Therefore, SIN may be promising as a new agent for the treatment of primary and metastatic cancers in humans.

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References

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