Intraperitoneal administration of an adenovirus vector carrying REIC/Dkk-3 suppresses peritoneal dissemination of scirrhous gastric carcinoma

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Abstract. Expression levels of the novel tumor suppressor gene REIC/Dkk-3 are reduced in many human cancers. We have previously showed that an adenovirus vector carrying REIC/Dkk-3 (Ad-REIC) induced apoptosis of cancer cells selectively and exerted bystander antitumor effects via ER stress. We examined possible effects of Ad-REIC in a peritoneal dissemination model of scirrhous gastric carcinoma (SGC). Among various types of gastric cancer, SGC continues to be associated with the worst prognosis due to a high incidence of metastases in the peritoneal cavity. We found that a single intraperitoneal injection of Ad-REIC suppressed tumor dissemination and disease progression. Immunomodulation by Ad-REIC led to recruitment of natural killer cells inside tumor nodules. We conclude that Ad-REIC gene therapy may be a potential tool in combinatorial approaches to achieve curative effects in SGC.

Introduction

Gastric cancer is the second leading cause of cancer death in men and the fourth in women in the world. The five-year survival rate in the United States and Europe is >25% (1). Among various types of gastric cancer, scirrhous gastric carcinoma (SGC) remains to be associated with the worst prognosis due to a high incidence of metastases in the peritoneal cavity. The five-year survival rate is 0-17% for SGC compared with 35-70% for other gastric carcinomas in Japan (2). Peritoneal metastasis is a critical limiting condition for the choice of treatment options to prolong patient survival. Once it occurs, most patients become inoperable.

Impairment of the immune surveillance system is known to contribute to cancer progression. Takeuchi et al (3) reported that activity of natural killer (NK) cells in gastric cancer patients was diminished and that there was a significant link between NK cell activity and tumor size. Tumor volume and dissemination were also affected by NK cell activity. Multimodal approaches using surgery and supportive therapies such as chemotherapy, radiotherapy, hormonal therapy and immunotherapy have been attempted. However, these combination therapies have not resulted in considerable improvement in the survival rate of SGC patients (4). Thus, development of new therapeutic tools to prevent or at least to suppress peritoneal metastasis is important for patients with SGC. The most critical challenge of SGC is dissemination in the peritoneal cavity, which results in formation of multiple cancer nodules. This precludes surgical removal of tumors and radiation therapy. Gene therapy therefore has a potential advantage since gene therapeutic agents can be applied directly into the peritoneal cavity.

We previously demonstrated that the tumor suppressor gene REIC/Dkk-3 was down-regulated in a number of human cancer cell lines (5). Overexpression of REIC/Dkk-3 gene using an adenovirus vector (Ad-REIC) had a dramatic therapeutic effect on human renal cell carcinoma (6), prostate cancer (7), breast cancer (8), testicular cancer (9) and pleural mesothelioma (10). Ad-REIC induced apoptotic cell death through activation of c-Jun N-terminal kinase (7-10). In addition, we have recently shown that Ad-REIC has a host-mediated bystander effect on human prostate cancer through IL-7 induction (11).

In the present study, we examined whether intraperitoneally administered Ad-REIC is effective for suppressing growth and dissemination of human SGC cells in a mouse model.
Materials and Methods

Animals. Female BALB/c nude mice at the age of 6-8 weeks were purchased from Charles River (Yokohama, Japan). All of the experiments were conducted in accordance with the guidelines for animal experiments of our institution.

Cell lines. A human scirrhous gastric cancer cell line, OCUM-2MD3 (12), and a prostate cancer cell line, PC3 (provided by American Type Culture Collection, Rockville, MD), were cultured in DMEM/F12 (Ham) (1:1) (Invitrogen, Carlsbad, CA). Scirrhous gastric cancer cell lines, 44As3 and 58As1 (13) were cultured in RPMI-1640 (Nissui, Tokyo, Japan). A normal human fibroblast cell line, OUMS-24 (14), was cultured in DMEM (Nissui). All media were supplemented with 10% fetal bovine serum (Invitrogen), 100 μg/ml kanamycin (Meiji Seika, Tokyo, Japan) and 0.5 μg/ml Fungizone (Invitrogen).

Adenovirus vectors. Ad-REIC was produced and propagated as described previously (7). An adenovirus vector carrying the LacZ gene (Ad-LacZ) was used for monitoring infection efficiency.

RT-PCR analysis. To extract total RNA, cells were processed by the acid guanidinium-phenol-chloroform method using TRI reagent (Sigma, St. Louis, MO). RT-PCR was performed under conventional conditions. The primers used were as follows: human REIC/Dkk-3 (forward) 5’-GGGGTGTGAACCATGAGAAGTATGA-3’, GAPDH (reverse) 5’-TGCTAAGCAGTTGGTGGTGTC-3’. Western blot analysis. Western blot analysis was performed under conventional conditions. The antibodies used were as follows: rabbit anti-human REIC/Dkk-3 antibody raised in our laboratory, mouse anti-human tubulin antibody (Sigma), and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA) as the second antibody.

Apoptosis assay. Cells were inoculated into flat-bottom 6-well plates and incubated for 24 h and then treated with Ad-LacZ or Ad-REIC at the indicated multiplicity of infection (moi). Seventy-two hours later, 1 μg/ml Hoechst 33342 (Invitrogen) and 5 μg/ml propidium iodide (Sigma) were added to the medium. Then the cells were incubated in the dark for 20 min. All of the cells were collected on a glass slide. Under a fluorescent microscope, cells with fragmented or shrunken nuclei were counted as apoptotic cells.

Intraperitoneal administration of Ad-REIC in a peritoneal dissemination model of SGC. OCUM-2MD3 cells (1x10⁷ cells in 200 μl PBS) were injected into the peritoneal cavity of female BALB/c nude mice at the age of 6-8 weeks. Twenty-four hours later, the mice were intraperitoneally injected with 4 ml PBS, 1x10⁶ plaque-forming units (p.f.u.) Ad-LacZ in 4 ml PBS, or 1x10⁹ p.f.u. Ad-REIC in 4 ml PBS. At different time points, mice were sacrificed and peritoneally disseminated cancer nodules were collected. The number of peritoneal nodules >1 mm in diameter was counted macroscopically. Cancer nodules were classified according to the longest...
diameter (<3, <3-5 and >5 mm). Tumor volume was calculated using an empirical formula, \( V = \frac{1}{2} \times (\text{shortest diameter})^2 \times (\text{longest diameter}) \). To examine the regional distribution of cancer nodules in the peritoneal cavity, the number of mice with at least one nodule in a given region was counted in each group.

**Immunohistochemical staining.** Collected tissues were fixed in 10% neutral-buffered formalin overnight. Paraffin sections of 6 μm in thickness were prepared. Terminal deoxy-nucleotidyltransferase-mediated UTP end-labeling (TUNEL) assay was performed using *In situ* Cell Death detection kit Fluorescein (Roche, Basel, Switzerland). Tissue sections were immunostained with Fluorescein isothiocyanate-conjugated rat anti-mouse CD49b antibody (Miltenyi Biotec, Auburn, CA) for identification of mouse NK cells under the conditions described previously (11).

**Results**

**In vitro effect of Ad-REIC/Dkk-3 on human scirrhous gastric cancer cell lines.** We first examined the endogenous expression level of REIC/Dkk-3 in human SGC cell lines (OCUM-2MD3, 44As3 and 58As1). Both mRNA and protein levels of REIC/Dkk-3 were down-regulated in scirrhous gastric cancer cells (Fig. 1A and B). Normal human fibroblasts (OUMS-24) expressed REIC/Dkk-3 protein with different molecular sizes due to differential glycosylation (Fig. 1B). The expression level of REIC/Dkk-3 after infection of OCUM-2MD3 cells with Ad-REIC was similar to that of PC3 cells (Fig. 1C).

Apoptotic rates of OCUM-2MD3 cells determined at 72 h after infection with Ad-REIC were 10 and 41% at the moi of 20 and moi of 100, respectively (Fig. 1D). Rates of Ad-REIC-induced apoptosis of PC3 cells, which are known to be sensitive to Ad-REIC, were 56 and 95% at 20 and 100 moi, respectively (Fig. 1D). Thus, OCUM-2MD3 cells showed limited sensitivity to induction of apoptosis by Ad-REIC in culture.

**Intraperitoneal administration of Ad-REIC suppresses dissemination and growth of SGC cells.** To explore the therapeutic efficacy of REIC/Dkk-3 gene transfer into the peritoneal cavity, OCUM-2MD3-inoculated mice were treated on day 1 with a single injection of Ad-REIC (10⁹ p.f.u./mouse) as shown in Fig. 2A. On days 7 and 14, the mice were sacrificed and tumor nodules were harvested (Fig. 2B and C). Ad-REIC-
treated mice on day 7 showed significantly lower tumor volume than did PBS or Ad-LacZ-treated mice. This trend was observed partially on day 14 (Fig. 3A).

On day 7, a significant tumor suppressive effect of Ad-REIC was obvious in smaller tumors of >5 mm (Fig. 3B). As shown in Table I, tumor incidence in Ad-REIC-treated mice than et al: REIC/Dkk-3 GENE THERAPY FOR SCIRRHOUS GASTRIC CARCINOMA

Table I. Incidence of dissemination in the peritoneal cavity at day 7.

<table>
<thead>
<tr>
<th>Region</th>
<th>PBS (n=5)</th>
<th>Ad-LacZ (n=5)</th>
<th>Ad-REIC (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sero-bloody/</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>bloody ascites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paragastric</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenic region</td>
<td>5/5</td>
<td>4/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Sub-diaphragm</td>
<td>4/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Kidney region</td>
<td>1/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Mesentery region</td>
<td>1/5</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Inguinal region</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Liver region</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>

aData are shown as the number of mice bearing at least one tumor (>1 mm) in each region.

Table II. Incidence of dissemination in the peritoneal cavity at day 14.

<table>
<thead>
<tr>
<th>Region</th>
<th>PBS (n=5)</th>
<th>Ad-LacZ (n=5)</th>
<th>Ad-REIC (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sero-bloody/</td>
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<td>5/5</td>
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<tr>
<td>Paragastric</td>
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<tr>
<td>region</td>
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<tr>
<td>Splenic region</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
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<tr>
<td>Sub-diaphragm</td>
<td>5/5</td>
<td>5/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Kidney region</td>
<td>5/5</td>
<td>5/5</td>
<td>1/5</td>
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<td>Inguinal region</td>
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<td>4/5</td>
<td>1/5</td>
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<tr>
<td>Liver region</td>
<td>0/5</td>
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</table>

aData are shown as the number of mice bearing at least one tumor (>1 mm) in each region.
was reduced particularly in splenic and subdiaphragmatic regions. Compared to the controls, dissemination to the paragastric or kidney region was also limited.

The average number of moderately sized (3-5 mm) tumors obtained from Ad-REIC-treated mice increased from day 7 to day 14 (Fig. 3B and C). However, this average number was still significantly less than that of the controls on day 14 (Fig. 3C). Additionally, as shown in Table II, there was a low incidence of tumors in mesentery, subdiaphragm, kidney and inguinal regions. Accumulation of sero-bloody or bloody ascites was also remarkably suppressed in Ad-REIC-treated mice.

Ad-REIC enhances recruitment of NK cells and apoptosis in tumor tissues. Immunohistochemical staining demonstrated TUNEL-positive cells inside tumor tissues of Ad-REIC-treated mice on day 7 (Fig. 4). In addition to the tumor suppressive

![Figure 4. Induction of apoptosis detected by TUNEL staining in tumor nodules examined on day 7. Bars, 200 μm.](image)

![Figure 5. Infiltration of NK cells detected by the specific marker CD49b in tumor nodules examined on day 7. Bars, 200 μm.](image)
termination through activation of NK cells. In the present study, disseminated SGC resulted in reduction of peritoneal dissemination mouse model, we observed the survival period in an SGC dissemination mouse model. To evaluate the overall effect of Ad-REIC administration in an SGC dissemination mouse model, we observed the survival period after treatment. Intraperitoneal inoculation of OCUM-2MD3 cells resulted in the formation of spheroid-like bodies in the peritoneal cavity. Intra-peritoneal administration of Ad-REIC prolonged survival in an SGC dissemination mouse model. To evaluate the overall effect of Ad-REIC administration in an SGC dissemination mouse model, we observed the survival period after treatment. Intraperitoneal inoculation of OCUM-2MD3 cells resulted in the formation of spheroid-like bodies in the peritoneal cavity.

In our study, both tumor tissues and the peritoneum were examined 4 days after injection (data not shown). Infection of normal cells with Ad-REIC resulted in suppression of peritoneal dissemination of OCUM-2MD3 cells and prolonged survival of cancer-bearing mice. However, Ad-REIC treatment alone was not sufficient to achieve high therapeutic efficacy in the peritoneal dissemination model of SGC we used. A challenge to explore the possibility of augmenting antitumor activity of REIC/Dkk-3 by combining with other therapeutic modalities remains to be pursued.

Acknowledgements

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References


Figure 6. The mean survival time of OCUM-2MD3-transplanted mice treated with Ad-REIC. Kaplan-Meyer analysis was applied for Ad-REIC-treated mice (n=10), Ad-LacZ-treated mice (n=9), and PBS-treated mice (n=10). P<0.005 by log-rank test.

Discussion

We previously demonstrated that Ad-REIC showed growth-suppressive and apoptosis-inducing effects on human cancer cells derived from the prostate, testis, pleura and breast (6-10). The induction of apoptosis was observed selectively in cancer cells and normal cells were marginally affected (7,9). When applied onto SGC, Ad-REIC efficiently infected and transduced cancer cells and normal cells were marginally affected (7,9).

Engler et al (23) reported that transgene expression was higher in the peritoneal wall than in tumor tissue when Ad-LacZ was injected into the abdominal cavity of nude mice bearing PC3 prostate cancer. In our study, both tumor tissues and the peritoneum were infected with Ad-LacZ as examined 4 days after injection (data not shown). Infection of both normal and malignant cells in the peritoneal cavity with Ad-REIC probably resulted in production of various cytokines and eventual activation of the host immune system.

Intra-peritoneal administration of Ad-REIC suppressed peritoneal dissemination of OCUM-2MD3 cells and prolonged the survival of cancer-bearing mice. However, Ad-REIC treatment alone was not sufficient to achieve high therapeutic efficacy in the peritoneal dissemination model of SGC we used. A challenge to explore the possibility of augmenting antitumor activity of REIC/Dkk-3 by combining with other therapeutic modalities remains to be pursued.

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