Trojan p16 Peptide Suppresses Pancreatic Cancer Growth and Prolongs Survival in Mice

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ABSTRACT

Purpose: The tumor suppressor gene p16INK4A is inactivated frequently in a large number of human cancers, and many investigators have attempted to restore the function of p16 using the p16 wild-type gene and viral vectors. In this study, we treated the tumor-bearing animals with the p16-derived synthetic peptide coupled with the Antennapedia carrier sequence, which we designated as Trojan p16 peptide.

Experimental Design: Injections (i.p.) of the Trojan p16 peptide (100 μg/mouse/day) were given for 3 weeks in the AsPC-1 and BxPC-3 s.c. tumor models. Tumor growth, histopathology, and TUNEL staining of the tumor and toxicity of the animals were evaluated. To examine its influence on the survival of tumor-bearing mice, Trojan p16 was administered in the AsPC-1 peritoneal dissemination model.

Results: In the AsPC-1 s.c. tumor model, a significant growth inhibition was obtained by the Trojan p16 treatment when compared with the three control treatments, i.e., vehicle, unconjugated form of p16, or Trojan peptide alone. Tumor growth inhibition was almost complete in the BxPC-3 tumor, a relatively slow growing tumor. Neither hematological cytotoxicity or body weight loss were observed. Histopathology of the BxPC-3 s.c. tumor in the Trojan p16 treatment group revealed marked vacuole formation and apoptotic death of cancer cells. In the AsPC-1 peritoneal dissemination model, the survival curve of mice treated with Trojan p16 was significantly longer than that of control.

Conclusions: These results provide evidence that the Trojan p16 peptide system, a gene-oriented peptide coupled with a peptide vector, functions for experimental pancreatic cancer therapy.

INTRODUCTION

The tumor suppressor gene p16INK4A, an inhibitor of cdk4, 4, is inactivated frequently through intragenic mutation, homozygous deletion, and methylation-associated transcriptional silencing in a large number of human cancers (1–3). Pancreatic cancer has the highest frequency of p16 alterations of all human malignancies (>95%; Ref. 4). The main biological function of the p16 involves regulation of the cell cycle, which blocks the transition through the G1-S phase in a pRb-dependent fashion by inhibiting cdk-4 and cdk-6 (5). Reconstitution of p16 gene in malignant cells revealed additional biological functions for p16, namely: (a) block of glioma cell invasion through MMP-2 inhibition (6); (b) down-regulation of vascular endothelial growth factor expression and inhibition of tumor angiogenesis in gliomas (7); (c) inhibition of α3β1 integrin-mediated cell spreading (8); and (d) induction of apoptosis on loss of anchorage (anoikis) via up-regulation of α5β1 fibronectin receptor (9). Thus far, many investigators have attempted to restore the function of p16 in a variety of tumors and obtained promising experimental results for future gene therapy using the p16 wild-type gene and viral vectors (6, 7, 9–13).

The amino acid residues of p16, which are important for its interaction with cdk4 and cdk6 and for the inhibition of pRb phosphorylation, were first demonstrated by Fahraeus et al. (14, 15). They identified a 20-residue synthetic peptide, corresponding to amino acids 84–103 of the p16 protein, that blocks cell cycle progression in late G1 in a pRb-dependent fashion. The p16 peptide was linked to the 16-amino acid Antennapedia homeodomain carrier sequence, which allowed the chimeric peptides to be transported across the membranes directly from the tissue culture medium to both the cytoplasm and nuclear compartments (16). The findings stimulate novel approaches for peptide-based cancer drug design that targets intracellular molecules (17).

To confirm whether this strategy is operative or not, we have investigated previously the effect of synthetic p16 peptide on the growth of pancreatic cancer cells in vitro (18). We found that the peptide with the Antennapedia carrier sequence was taken up smoothly by the cells when they were cultured in serum-containing media and subsequently inhibited the growth of p16-negative and pRb-positive pancreatic cancer cells, inducing G1 phase cell cycle arrest through the inhibition of pRb phosphorylation. The results were expected; however, there has been no evidence that this strategy is operative in vivo.
been no report demonstrating that this type of chimeric peptide also functions in *in vivo* circumstances. In the current study, we treated the tumor-bearing animals with the p16-derived synthetic peptide coupled with the Antennapedia carrier sequence, which we designated as Trojan p16 peptide (19). We found that systemic treatment with Trojan p16 peptide suppressed pancreatic cancer growth and prolonged survival. The p16 peptide coupled to a nonviral vector may serve as an alternative therapy for the pancreatic cancer through restoration of p16/pRb tumor-suppressive pathway.

**MATERIALS AND METHODS**

**Peptides, Cells, and Mice.** A 21-residue synthetic peptide corresponding to amino acids 84–103 + Cysteine (DAAREGFLDLTVVLHRAGAR-Cys) of the p16 protein (14, 15) was synthesized (A. O. and N. F.). A 17 amino acid region of the Antennapedia homeodomain (designated as Trojan peptide; Cysteine + amino acid 43–58: Cys-RQIKIWFQNRMKWKK) was also synthesized. The Trojan peptide was then linked to the COOH terminus of the p16-derived synthetic peptide (amino acid, 84–103) by a disulfide bridge and was designated as Trojan p16 peptide. For Trojan alone injection, the NH₂ terminus of the peptide was capped chemically to avoid the possible uptake of nonspecific substances. These synthetic peptides were initially dissolved in DMSO at a concentration of 10 mg/ml and stored in the dark at −80°C.

Two human pancreatic cancer cell lines, AsPC-1 and BxPC-3, were obtained from American Type Culture Collection (Rockville, MD). The cells were grown as monolayers in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂. These cell lines were chosen for this *in vivo* study, because we had demonstrated previously that these cells were negative p16 protein expression and responsive to Trojan p16 treatment *in vitro* (18).

Four-week-old male BALB/c- nu/nu mice (SLC, Hamamatsum, Japan) were used. They were maintained and fed in sterile conditions and fed with sterile food and water *ad libitum*. All procedures involving animals and their care were carried out at the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, in accordance with institutional guidelines.

**Animal Models and Treatment Schedule.** Cells in the exponential growth phase were detached with EDTA and washed twice in PBS. Cell number and viability were assessed, and cell cultures with a viability > 90% were used. For the s.c. tumor model, AsPC-1 or BxPC-3 cells (2 × 10⁶; 200 µl in RPMI) were injected i.c. into the back of athymic mice. For the peritoneal dissemination model, AsPC-1 (3 × 10⁶; 200 µl) was injected into the peritoneal cavity of mice.

As for the s.c. tumor model, Trojan p16 treatment was injected i.p. at a dose of 100 µg/mouse/day for 5 consecutive days, and after 2 days off, it was administered for 3 weeks. Trojan p16 treatment was repeated in a separate s.c. model, namely, mice bearing a rapid growing AsPC-1 tumor (n = eight in each treatment group) and mice bearing a BxPC-3 tumor with a moderate growing rate (n = four in each group). When the s.c. tumor was clearly visible, Trojan p16 treatment was started; it was 10 days after implantation in the AsPC-1 s.c. tumor model and it was 14 days after implantation in the BxPC-3 s.c. tumor model. As the control treatment, the vehicle (DDW) was given in the same manner as Trojan p16 treatment. To examine the specific effect of the Trojan p16 peptide, two control treatments, *i.e.*, the unconjugated form of Trojan peptide or p16 peptide, were administered at a dose of 50 µg/mouse to the AsPC-1 s.c. tumor model; the dose was equivalent to that of Trojan p16 peptide on a molar basis.

As for the AsPC-1 peritoneal dissemination model (n = 12 in each group), Trojan p16 treatment was repeated in separate experiment with a lower dose of 100 µg/mouse/day and a higher dose of 200 µg/mouse/day. The treatment was started 24 h after the i.p. injection of the cancer cells and continued as the same manner as the s.c. tumor model.

**Measurements and TUNEL Staining.** In the s.c. tumor model, tumor growth was monitored at 7-day intervals by measuring the two orthogonal diameters (D, the longer and d, the shorter) of the tumor mass with a caliper and by applying the formula D × d × d/2 to calculate the tumor volume. Later (21 days), the animals were sacrificed, blood was drawn for blood cells counts, and the tumor was excised, weighed, and fixed in 4% paraformaldehyde in PBS. In the peritoneal dissemination model, the end point of the experiment was the survival period of the animals.

TUNEL assay was undertaken to assess apoptotic cell death in the s.c. tumor; details of the procedure were described previously (20). In brief, tissue samples were embedded in paraffin and cut into 4-µm-thick consecutive sections. The serial sections were deparaffinized in three changes of xylene, rehydrated in descending concentrations of ethanol, and then washed three times for 5 min each with DDW. After rehydration, the sections were treated with 20 µg/ml proteinase K (Oncor, Gaithersburg, MD) at 37°C for 15 min and then washed in DDW. After proteinase K treatment, the sections were immersed in TDT buffer (30 mM Trizma base, 140 mM sodium cacodylate, and 1 mM cobalt chloride), then incubated with TDT buffer containing 12.5 µM biotinylated dUTP (Boehringer Mannheim, Mannheim, Germany) and 0.15 units/µl TDT (Takara, Kyoto, Japan) at 37°C for 70 min. The reaction was terminated by immersing them in terminating buffer (300 mM sodium chloride and 30 mM sodium citrate). The sections were incubated in streptavidin-peroxidase complex for 30 min and then developed with diamobenzidine-tetra-hydrochloride for 1–5 min as a substrate. As positive control samples, the ovaries of adult mice were subjected to TUNEL assay, and positive TUNEL labeling was confirmed in the nuclei of follicular cells in the maturing follicles (20). The number of TUNEL-positive cells in the sample was counted, and the apoptotic index was calculated as described previously (20).

**Statistical Analysis.** Data are expressed as the mean value ±SE. Statistical differences among treatment groups of the s.c. tumor model were assessed by the Wilcoxon nonparametric comparison test. The Kaplan-Meier method was used to calculate survival curves of treatment groups of the peritoneal dissemination model, and Log-rank and generalized Wilcoxon tests were performed to compare differences in survival rates. All statistical analyses were done using JMP statistical software.
RESULTS

Inhibition of Tumor Growth in the s.c. Tumor Model.
To examine the inhibitory effect on tumor growth, the Trojan p16 peptide was administered systemically to mice bearing AsPC-1 s.c. tumor and compared with three control treatments, namely, vehicle (DDW) alone, p16 peptide alone, and Trojan peptide alone. The tumor growth curve of animals treated for 3 weeks with p16 peptide alone (50 μg/mouse/day) or the Trojan peptide alone was similar to that observed in mice treated with vehicle. The tumor growth curve in mice treated with the Trojan p16 peptide (100 μg/mouse/day) was reduced significantly when compared with that of the p16 peptide alone group, Trojan peptide alone group, or vehicle treatment group (Fig. 1A). When animals were sacrificed at 21 days after the start of the treatment, the weight of the excised tumor was 139 ± 16 mg in the vehicle group, 151 ± 15 mg in mice treated with the p16 peptide alone, 149 ± 12 mg in mice treated with the Trojan peptide alone, and 79 ± 17 mg in mice treated with the Trojan p16 peptide (Fig. 1B). Inhibition of tumor growth by treatment with Trojan p16 was statistically significant (P < 0.05, Wilcoxon nonparametric comparison test).

Instead of challenging the dose-response study, we repeated the experiment with the same dose schedule in a relatively slow growing tumor model, the BxPC-3 s.c. tumor model. Because the specific effect of Trojan p16 was demonstrated in the AsPC-1 model, vehicle treatment served as the control in this case. There was a marked difference in the results obtained with the AsPC-1 and BxPC-3 s.c. models. In BxPC-3 models, the tumor growth curve in mice treated with the Trojan p16 peptide was almost completely inhibited when compared with that of control group (Fig. 2A). The tumor wet weight in the BxPC-3 s.c. tumor model was 41 ± 13 mg in the control group, which was ~30% of that of the AsPC-1 tumor. The tumor wet weight (6 ± 2 mg) in mice treated with Trojan p16 was significantly smaller than control (Fig. 2B).

Histopathological Changes in the s.c. Tumor. Histopathological changes were evaluated in BxPC-3 tumors removed on day 21 of treatment. H&E staining demonstrated marked vacuole formation inside the tumor in mice treated with Trojan p16 (Fig. 3B). When the tumor samples were subjected to TUNEL staining, substantial numbers of TUNEL labeling were detected in the nuclei of cancer cells lining the vacuole (Fig. 3D). These histopathological changes were not observed in the control tumor samples (Fig. 3, A and C). The apoptotic index of s.c. tumor treated with Trojan p16 was significantly higher than that of control (16 ± 4% versus 4 ± 1%, P < 0.05 by Wilcoxon nonparametric comparison test).

In the separate experiment, we have treated the BxPC-3 s.c. tumor model with control saline injections for 4 days, and the tumor was harvested and subjected to H&E and TUNEL staining. Neither vacuole formation nor TUNEL-positive nuclei were detected in the control small tumor whose size was equivalent to that of tumors in mice treated with Trojan p16.
Trojan p16 Peptide Inhibits Pancreatic Cancer Growth

To examine its influence on the survival of tumor-bearing mice, Trojan p16 was administered to mice in the AsPC-1 peritoneal dissemination model (Fig. 4). All mice died of cachexia associated with either massive ascites or bowel obstruction, regardless of the type of the treatment. The survival curve of the mice treated with 200 µg/mouse/day Trojan p16 peptide was significantly better than control, with a survival benefit of 6 days in terms of mean survival time. When the lower dose (100 µg/mouse/day) was administered in the same model, there was no significant survival benefit (Table 2).

### Table 1: Changes in body weight and peripheral blood cell counts of animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Body weight (grams)</th>
<th>Peripheral blood cell count (day 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>RBC ($\times 10^6$)</td>
</tr>
<tr>
<td>AsPC-1 S.C. tumor model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>23.5 ± 0.3</td>
<td>974 ± 20</td>
</tr>
<tr>
<td>p16 alone</td>
<td>8</td>
<td>23.3 ± 0.4</td>
<td>963 ± 15</td>
</tr>
<tr>
<td>Trojan alone</td>
<td>8</td>
<td>23.9 ± 0.2</td>
<td>998 ± 22</td>
</tr>
<tr>
<td>Trojan p16</td>
<td>8</td>
<td>22.9 ± 0.3</td>
<td>993 ± 22</td>
</tr>
<tr>
<td>BxPC-3 S.C. tumor model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>22.6 ± 0.3</td>
<td>978 ± 38</td>
</tr>
<tr>
<td>Trojan p16</td>
<td>4</td>
<td>23.3 ± 1.0</td>
<td>942 ± 20</td>
</tr>
</tbody>
</table>

* PLT, platelet counts.

**DISCUSSION**

The current study demonstrated in mice s.c. tumor models that i.p. injections of the Trojan p16 peptide significantly inhibited pancreatic cancer growth without exhibiting severe systemic toxicity. The extent of inhibition varied between the rapid- and the moderate-growth tumors, but no inhibition was obtained with the unconjugated form of the p16 peptide. Histopathology of the s.c. tumor in the Trojan p16 treatment group revealed marked vacuole formation and apoptotic death of cancer cells. Trojan p16 also contributed to a modest but statistically significant survival benefit observed in the peritoneal dissemination model of pancreatic cancer.

The Trojan peptide is a 16-mer oligopeptide derived from the homeodomain of Antennapedia, a synonym of penetratin, and it is reported to enter cells via a nonendocytotic and receptor- and transporter-independent pathway, even when conjugated with large hydrophilic molecules (16, 19, 21). The exact mechanism of this cell-penetrating peptide is still unknown, but recent studies indicate that the COOH-terminal segment of seven amino acid residues is essential for plasma membrane translocation (21) and that the uptake mechanism depends on the direct interaction of the peptide with the lipid bilayer of the plasma membrane (22, 23). This system allows direct targeting of oligopeptides and oligonucleotides to the cytoplasm and nucleus and has applications of potential cell biology and clinical interest as an efficient transduction vector (19, 24). A number of oligopeptides has been developed as a cargo and proved to function against cancer cells with the help of this peptide vector. These are the p53 COOH-terminal peptide (25, 26), p21WAF1-derived peptide (27), p16INK4A-derived peptides (14, 15, 18), BH3 domain peptide of Bak that antagonizes Bcl-XL (28), Sos-derived peptide that blocks Ras-signaling (29), and the c-Myc derived peptide that interferes with c-Myc transcription activity (30). The results are challenging for future cancer therapy; however, all of the studies were performed in vitro. Recently, the Trojan peptide has been reported to cross the blood-brain barrier of experimental animals after administration through an in situ brain perfusion system and by i.v. injection (22). To our knowledge, the current study is the first report to demonstrate that systemic treatment with the Trojan p16 peptide, a gene-oriented peptide coupled with a peptide vector, suppresses cancer growth in experimental animals.

The Trojan peptide shows highly efficient internalization and no cell-type specificity (19). Our previous in vitro study...
demonstrated that the Trojan p16 peptide was taken up by osteosarcoma cells and fibroblasts, as well as pancreatic cancer cells. However, the cell cycle arrest and subsequent growth inhibition elicited by Trojan p16 were observed in p16-negative and pRb-positive pancreatic cancer cells but was not observed in p16-positive and pRb-negative osteosarcoma cells or fibroblasts positive for both p16 and pRb (18). Because i.p. injections of Trojan p16 resulted in a significant growth inhibition of s.c. tumors in the current study, the peptide is likely to be delivered into the tumor through the systemic circulation. The peptide might penetrate noncancerous quiescent or proliferating cells through the systemic circulation, but neither hematomatous cytotoxicity or body weight loss were observed at the dose levels used in this study. In a separate experiment, we tried to identify the uptake of this peptide by the s.c. tumor and systemic organs in mice. Biotinylated Trojan p16 was injected i.p., and tissue samples were subjected to FITC-avidin staining, but FITC fluorescence was under detectable levels (data not shown). Although direct evidences of the peptide delivery were not provided in the current study, the results suggest that cell-type-specific status of p16 deficiency might be responsible for the effect of p16 treatment.

Control of the cell cycle at G1–S boundary is not the sole biological function of p16INK4A. Plath et al. (9) showed that stable transfection of the p16 gene represses apoptosis induction on loss of anchorage (anoikis) in pancreatic cancer cell lines and indicated that p16 might influence a critical feature of a malignant epithelial phenotype, anchorage dependence. They also demonstrated, in mice, that p16-transfected cell lines exhibited remarkably reduced tumorigenicity and cellular apoptosis, as detected by TUNEL assay. Our previous in vitro p16 treatment did not cause apoptosis but G1 arrest when cultured under anchorage-dependent conditions (18). Although the specific mechanisms have not been determined, apoptotic cancer cell death observed in s.c. tumors might reflect the other function of p16 in vivo.

With the use of the p16 peptide, Fahraeus et al. (8) demonstrated another biological function of p16, i.e., inhibition of αvβ3 integrin-mediated cell spreading. Integrins are important molecules that regulate adhesion, migration, and invasion of cancer cells through interaction with the extracellular matrix and are involved in peritoneal carcinomatosis (31). We have shown previously that invasiveness of pancreatic cancer was related greatly to MMP-2 activation and αvβ3 integrin expression and that AsPC-1 cells had the most invasive phenotype with the strongest expression of MMP-2 and αvβ3 integrin (32, 33). The prolonged survival of the mice treated with Trojan p16 in the AsPC-1 peritoneal dissemination model might be because of suppression of invasiveness-related molecules, as well as inhibition of cancer growth. Because the p16 tumor-suppressive pathway is abrogated in virtually all pancreatic carcinomas (4) and this tumor is characterized by aggressive local invasiveness and metastasis (34), p16 might be one of the best target molecules for gene-oriented therapy for pancreatic cancer.

Gene-oriented therapy involves the transfer of gene constructs, oligonucleotides, and peptides, and the vectors used in the transfer are generally viruses. Various problems have been encountered in their use, including transfer efficacy, complex manipulation, cellular toxicity, and immunogenecity, which would preclude their routine use in vivo (35). The Trojan peptide and other internalization peptides (24, 35) might be the alternatives. Our current study provides evidence that the Trojan p16 peptide system functions for experimental pancreatic cancer therapy. The maximal tolerance doses, tissue and blood distribution of the peptide, and unpredictable toxicity, such as anaphylaxis of the polypeptide, should be determined in the future preclinical study. Moreover, the efficacy and the mechanism of the action of this peptide therapy must be compared with those of p16 gene transfer using viral vectors.

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