A new molecular targeted therapeutic approach for renal cell carcinoma with a p16 functional peptide using a novel transporter system

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Abstract. Molecular targeting agents have become formidable anticancer weapons showing much promise against refractory tumors and functional peptides and are among the more desirable of these nanobio-tools. Intracellular delivery of multiple functional peptides forms the basis for a potent, non-invasive mode of delivery, providing distinctive therapeutic advantages. We examine the growth suppression efficiency of human renal cell carcinoma (RCC) by single-peptide targeting. We simultaneously introduced $p16^{INK4a}$ tumor suppressor peptides by Wr-T-mediated peptide delivery. Wr-T-mediated transport of $p16^{INK4a}$ functional peptide into 10 RCC lines, lacking expression of the $p16^{INK4a}$ molecule, reversed the specific loss of p16 function, thereby drastically inhibiting tumor growth in all but 3 lines by >95% within the first 96 h. *In vivo* analysis using SK-RC-7 RCC xenografts in nude mice demonstrated tumor growth inhibition by the $p16^{INK4a}$ peptide alone, however, inoculation of Wr-T and the $p16^{INK4a}$ functional peptide mixture, via the heart resulted in complete tumor regression. Thus, restoration of tumor suppressor function with Wr-T peptide delivery represents a powerful approach, with mechanistic implications for the development of efficacious molecular targeting therapeutics against intractable RCC.

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Introduction

Renal cell carcinoma does not respond to chemotherapy or radiation therapy. In addition, unresectable recurrences or metastases have been treated only by cytokine therapy with INF or IL-2, which is not so effective, with a response rate of about 10-20% (1-6). Recently, tyrosine-kinase-targeting inhibitors (such as sorafenib, sunitinib, everolimus and temsirolimus), which are involved in the growth of cancer cells, and other signal transduction inhibitors have been developed for use in molecular targeted therapy and are beginning to be indicated for the treatment of metastatic renal cancer (7-12). Although these molecular targeting drugs are more effective than cytokine therapy, they have not provided satisfactory therapeutic results. Moreover, tyrosine-kinase inhibitors are associated with adverse reactions, including hand-foot syndrome and hypertension, while m-TOR inhibitors may cause adverse reactions such as interstitial pneumonia. Therefore, there is a need for the continued development of more effective therapeutic agents associated with fewer adverse reactions for the treatment of renal cell carcinoma.

Protein transduction domains (PTDs) have recently been receiving attention as safe and effective tools in intracellular drug delivery systems. PTDs, being able to pass through the cell membrane of living cells, are considered useful for intracellular delivery of functional proteins or peptides targeting intracellular molecules, and many PTDs, including HIV-1 TAT, pAntp43-58 and polyarginine (R4-16), have been reported (13-23). In 1998, Nagahara *et al* produced a recombinant protein (TAT-$p27^{kipl}$ fusion protein), transduced it into cells, and induced G1 arrest and cell migration (16). Kondo *et al* developed a system through which functional peptides and their transporter peptides were synthesized separately and attached to each other by mixing into a solution, and the

Table I. PCR primers and condition.

Molecule	Sequences	Annealing temperature (˚C)	Fragment (bp)
p16	F: ATAGTTACGGTCGGAGGCC R: TGGTTACTGCCTCTGGTGC	60	536
Cyclin D	F: AAAGACAGTTTTTGGGTAATCTTTT R: CCGGAGCATTTTGATACCAG	55	126
CDK4	F: CTTCTGGACACTGAGAGGGC R: TGGGAGGGGAATGTCATTAA	61	110
CDK6	F: CGGAGAACACCCTTGGTG R: GAGCCTGTCCAGAAGACAGC	59	105
Actin	F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAATGTCACGCACGATTTC	55	539

resulting complex was then used to deliver functional peptides to cells, instead of a system through which PTDs were directly bound to functional peptides or proteins. This transporter, Wr-T, consisted of tryptophan-rich domains (serving as a cargo to attach functional peptides) fused with nine D-arginines serving as a PTD. They reported that the Wr-T system provided higher delivery efficiency than Pep-1 (whose delivery efficiency is already reported) and achieved an antitumor effect by delivering antitumor peptides to leukemia, lymphoma and glioma cells (21-23). In the present study, we delivered antitumor peptides to renal cell carcinoma cell lines using a similar system, and evaluated their antitumor effect. The antitumor peptides showed a strong antitumor effect on renal cell carcinoma without causing any abnormalities in normal tissues. This finding indicates that this therapy, associated with few adverse reactions, shows promise for clinical application in cancer treatment.

Materials and methods

Cells. Human RCC lines SK-RC-1, 6, 7, 12, 14, 17, 33, 44, 52 and 59 (kindly provided by Dr Lloyd J. Old, MSKCC), human cervical cancer cell line (HeLa) and human bladder cancer cell line (575A) were maintained in RPMI-1640 containing 10% inactivated fetal bovine serum (IBL, Gunma, Japan), 100 U/ml of penicillin and 0.1 mg/ml of streptomycin, at 37˚C under an atmosphere of 5% CO_2.

Peptide synthesis. All peptides including Wr-T, r9-p16 minimal inhibitory sequence (MIS) were synthesized at BioGate Co. Ltd. (Yamagata, Japan). The identity of all peptides was confirmed by mass spectrometry. We prepared the HCl form of the peptides following high-performance liquid chromatography purification for *in vitro* and *in vivo* applications. Peptide purity was >95%. The amino acid sequence of the Wr-T transporter is: KETWWETWWTEWWTEWSQ GPGrrrrrrrrr (r, D-enantiomer arginine) (21,22). For the synthesis of p16 MIS, the 10 sequential amino acid residue sequence 'FLDTLVVLHR', identified as the MIS of p16 by Fahraeus *et al* (24), was defined as the functional core of the peptide, which is insoluble, as is the entire p16 molecule (MIS hydrophobicity, 69.2%). We therefore fused r9 to these 10 amino acids to make the conjugate less hydrophobic (hydrophobicity, 40%), thus facilitating incorporation into the cells.

Peptide transduction. For the incorporation of the peptide mixture for *in vitro* growth suppression, the Wr-T and r9-p16 MIS peptides were mixed in 10 μl of distilled water at room temperature for 60 min (final concentration: Wr-T, 5 μmol/l; r9-p16 MIS, 8 μmol/l). The solution was then added directly to 190 μl of RPMI-1640 containing 5% fetal bovine serum to obtain the indicated final concentration. *In vivo* peptide delivery to solid human RCC was performed as follows: the Wr-T/r9-p16 MIS peptide mix (Wr-T, 50 nmol; r9-p16 MIS, 80 nmol) was injected into the hearts of mice bearing tumors that had grown to a diameter of 5 mm (tumor volume, ~150 mm^3). Control groups were done in parallel by administering 100 μl of PBS without peptide, Wr-T or p16 peptide alone dissolved in 100 μl of PBS and injected as previously described (22).

Flow cytometry. Cell cycle analysis was carried out using FACSCanto (BD, Franklin Lakes, USA) on cells whose DNA was stained with 10 mg/ml propidium iodide 24 h after the introduction of the peptides, according to the manufacturer's staining protocol (cell cycle analysis, GeneScript, Piscataway, NJ, USA). Apoptosis assays were performed using the FITC-Annexin V staining kit (MBL, Ina, Japan) on peptide-treated cells followed by FACSCanto analysis.

Reverse transcription PCR. Five micrograms of total RNA was extracted from each SK-RC/575A cell line using RNaesy mini (Qiagen, Valencia, CA, USA). Subsequently, cDNA was synthesized from the extracted RNA using random primers and a cDNA synthesis kit (High Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA USA). Reverse transcription-PCR was then carried out with Taq polymerase (Ampli-Taq Gold, Applied Biosystems). Amplification conditions and primer sequences are listed in Table I. The sense/antisense primer sequences for CDK4, CDK6 and Cyclin D were as described previously (25).

Western blotting. Cells were promptly lysed with SDS sample lysis buffer and the extracts were separated by SDS-PAGE using 12.5-15% bis-Tris gradient gels (SuperSepAce, Wako, Osaka,

Japan). Proteins were transferred onto a PVDF-membrane (Immobilon-P, Millipore, Billerica, MA, USA), blocked with 5% dried milk and 1% normal goat serum-PBS and then sequentially probed with the following antibodies: mouse monoclonal anti-$p16^{INK4}$ antibody (Clone: G175-1239, BD Biosciences Pharmingen, San Diego, CA, USA), mouse monoclonal anti-$p21^{WAF1}$ antibody (Clone: EA10, Oncogene Research Products, Boston, MA, USA), mouse monoclonal anti-$p27^{Kip1}$ antibody (Clone: 1B4, Novocastra, Newcastle, UK), mouse monoclonal anti-RB antibody (Clone: LM95.1, Oncogne), rabbit polyclonal anti-phospho-Ser^{780} pRB antibody (Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-actin antibody (Clone: AC-74, Sigma, St. Louis, MO, USA). Immune-complexes were visualized with the ECL plus Western Blotting Detection System (RPN2132, Amersham Pharmacia Biotech UK Limited, UK) according to the manufacturer's instructions and signals were visualized and digitally captured using an image analyzer (LAS 1000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Mouse tumor models. Four-week-old KSN female nude mice were obtained from SLC, Inc. (Hamamatsu, Japan). A 40-μl of RPMI-1640 suspension containing $2.0x10^6$ cells of the human RCC line, SK-RC-7, was injected s.c. into the flanks of each mouse to form a solid tumor nodule. Animal experiments performed in this study were approved by the Aichi Medical University Subcommittee on Animal Research. All mouse procedures, euthanasia and surgery, including renal cell cancer transplantations and peptide injections, were done painlessly or under anesthesia, within the strict guidelines of the Experimental Animal Facility of Aichi Medical University.

Apoptosis analysis. Detection of apoptotic cells were detected in tumors harvested from mice 48 h after the peptide administration. Tumors were fixed in 10% neutral buffered formalin overnight and were then processed, paraffin embedded, sectioned and mounted onto slides. Apoptosis in the tumor sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay method with the use of the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Tokyo, Japan) following the manufacturer's instructions.

Results

INK4 family tumor suppressor background in RCC. We first examined the mRNA and protein expression status of p16 and several key molecules associated with the cell cycle from 10 RCC lines. Immunoblotting analysis showed that none of the 10 RCC lines expressed the p16 protein product, but expressed p27 protein, and 6 out of 10 cell lines expressed p21 protein, all renal cancer cell lines expressed the phosphorylated form of the pRB protein (Fig. 1A). Consistent with this non-expression of the p16 molecule, multiple forms of phosphorylated pRB, including Ser^{780}, were detected in these cells, indicating pRb activation along with accelerated cell proli-feration. In addition, RT-PCR analysis detected Cyclin D, CDK4 and CDK6 in RNAs from all 10 RCC lines, but not p16 (Fig. 1B).

Growth inhibition of RCCs by the Wr-T/r9-p16 transduction system. Based on these results, we attempted to suppress the growth of RCC cells by using the Wr-T-transported r9-p16 MIS. We introduced r9-p16 MIS into this background by mixing each RCC line with Wr-T (final concentration: Wr-T, 5 μmol/l; r9-p16 MIS, 8 μmol/l) and monitoring cell proliferation, starting with $4.0x10^4$ cells per incubation. Administration of r9-p16 MIS alone showed some growth suppression in 7/10 cell lines. However, all tumor lines incubated with the Wr-T/r9-p16 MIS mixture showed drastic suppression of cell proli-feration (Fig. 2). At 24 h post-transduction, FACS analysis with propidium iodide staining

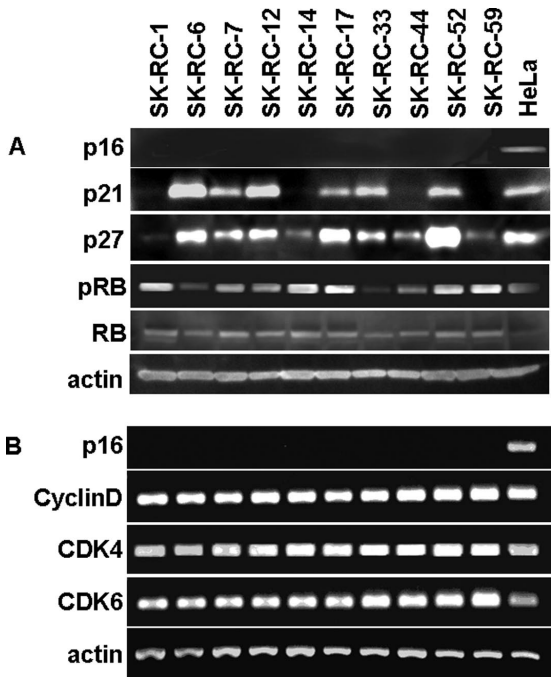

Figure 1. Loss of $p16^{INK4a}$ expression was correlated to cell cycle pathway genes in 10 human renal cell carcinoma cell lines by Western blotting and RT-PCR. (A) Endogenous protein expression of $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$ RB and phospho-Ser^{780} pRB in the examined cell lines by immunoblotting. (B) endogenous mRNA expression of $p16^{INK4a}$, Cyclin D, CDK4, CDK6 and actin by reverse transcription-PCR in 10 human renal cell carcinoma cell lines, SK-RC-1, 6, 7, 12, 14, 17, 33, 44, 52 and 59.

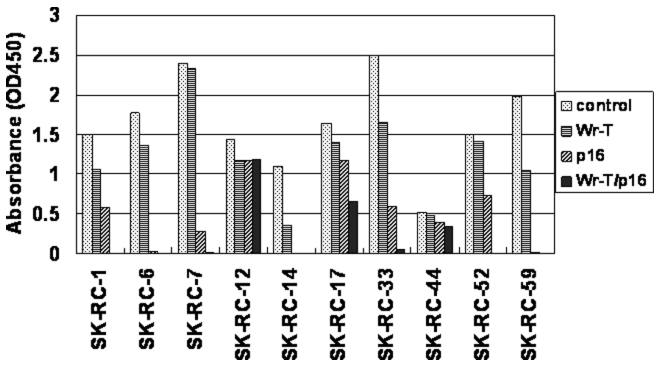

Figure 2. Cell growth suppression by transduction of Wr-T and p16 peptides. Ten representative cases of renal cell carcinoma cell lines are shown. Proliferation of cells treated with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 8 μmol/l) was compared with cells treated without peptides, with Wr-T (5 μmol/l) alone and with p16 (8 μmol/l) alone using WST-1 assay.

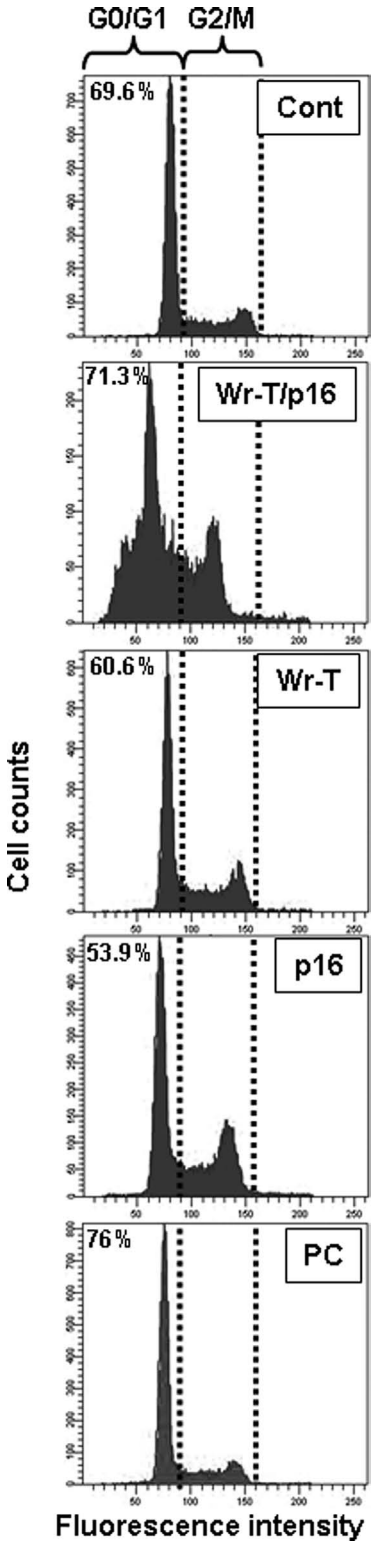

Figure 3. Enlarged G0-G1 phase in renal cell carcinoma cells treated with Wr-T and p16 peptides mixture. Cell cycle profiles of propidium iodide stained SK-RC-7 cells transduced with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 6 μmol/l), without peptides, with Wr-T (5 μmol/l) alone, and with p16 (6 μmol/l) alone 24 h after treatment. Percentage of cells in G0-G1 phase is indicated.

showed that SK-RC-7 cells incubated with the Wr-T/r9-p16 MIS mixture preferentially accumulated at the G0-G1 (71.3%) phase, compared with mock-treated cells (69.6%) and cells

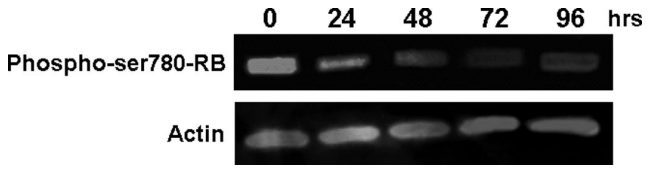

Figure 4. Reduced expression of phospho-Ser^{780} pRB in Wr-T/r9-p16 MIS-introduced cells assessed by immunoblotting (Wr-T, 5 μmol/l; p16, 8 μmol/l).

treated only with r9-p16 MIS (53.9%) (Fig. 3). This result was similar to that obtained with cells treated with staurosporin, a G0-G1 cell cycle inhibitor (26). Because arrest in G1 phase is expected to abrogate phosphorylation of pRB, we investigated the practical effect that the Wr-T/r9-p16 MIS mixture would have on pRB status. By 72 h post-transduction, phospholylated pRB was dramatically decreased in cells containing the Wr-T/r9-p16 MIS mixture, which is consistent with the induction of cell cycle arrest effected by the newly introduced p16 MIS (Fig. 4). About 52% of these cells were Annexin V positive at 24 h post-transduction, suggesting that the nuclear r9-p16 MIS had triggered apoptosis (Fig. 5).

In vivo RCC tumor suppression by the Wr-T/r9-p16 transduction system. Because of the therapeutic potential of the Wr-T/r9-p16 MIS delivery system, we tested the efficacy of this system for the treatment of the human RCC line, SKRC-7, xenografts transplanted subcutaneously in KSN nude mice. When tumors grew to 5 mm, we administered the Wr-T/r9-p16 MIS mixture into the mice via cardiac delivery. A clear decrease in tumor size was evident after the first ten days in the mice treated with a single dose of the Wr-T/r9-p16 MIS mixture, whereas the peptide free tumors grew to twice their initial size. In addition, sustained suppression of tumor growth continued to 28 days in mice treated with three doses of the Wr-T/r9-p16 MIS mixture, though tumor growth eventually resumed in mice receiving only a single dose (Fig. 6A and B). At 48 h post-transduction, TUNEL analysis showed an increase in the presence of positively stained apoptotic bodies in tumor treated with the Wr-T/r9-p16 MIS mixture (Fig. 6C), suggesting that the nuclear incorporation of the r9-p16 MIS had triggered apoptosis.

Discussion

Cancer cells grow abnormally, probably because they have escaped the cell cycle control present in normal cells. One example is cell cycle progression caused by overexpression of Cyclin D1 due to translocation or amplification of the PRAD1 gene (27). Another example is cell cycle progression mediated by cyclin (which does not work in normal cells) due to the absence of cyclin inhibitors, such as p16^{INK4a} and p27^{Kipl} (28). Several reports are available on the expression of molecules involved in the cell-cycle process in patients with renal cell carcinoma. Ikuerowo *et al* analyzed the expression of p16^{INK4a} in tumor samples from renal cell carcinoma using immunostaining, and observed absent or low expression of this gene in 82% of samples (29). Scharml *et al* performed microarray analysis and reported cyclin-dependent kinase inhibitor 2A

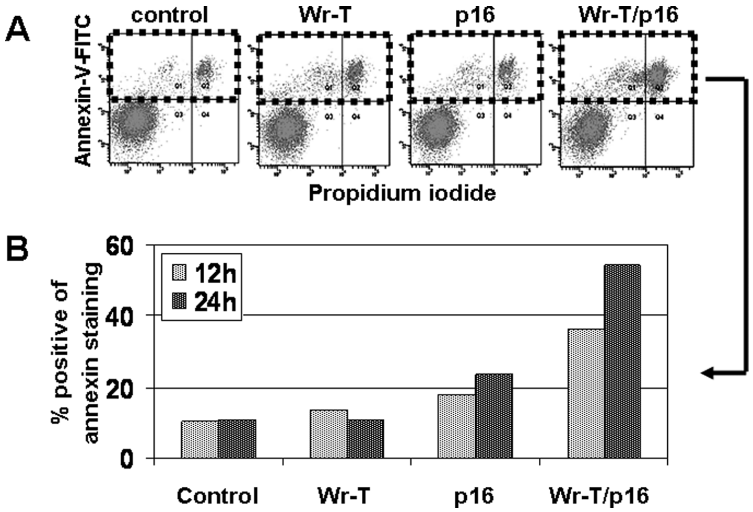

Figure 5. Apoptotic induction of renal cell carcinoma cells treated with Wr-T and p16 peptides mixture. The SK-RC-7 cells were treated with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 8 μmol/l), without peptides, with Wr-T (5 μmol/l) alone, and with p16 (8 μmol/l) alone. (A) Flow cytometry profiles of live, apoptotic and necrotic cells at 12 and 24 h after the treatment by staining with Annexin V-FITC and propidium iodide. (B) Columns, percentages of Annexin V-positive cells in each indicated sample from (A).

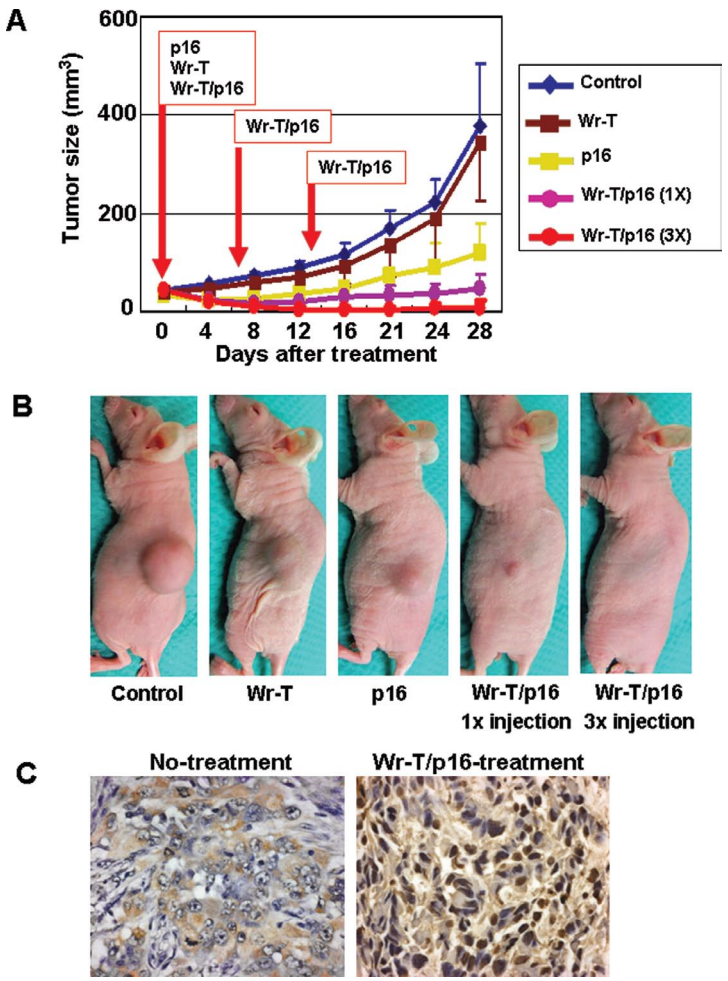

Figure 6. Effects of *in vivo* administration of Wr-T and p16 peptides mixture on tumor growth. SK-RC-7 cells ($1x10^6$) were transplanted into the backs of the nude mice. Treatments were performed as described in Materials and methods using 5 mice in each group. (A) Growth curves of tumors. Sizes of the tumors were determined by first measuring length (L) and width (W) and then the tumor volume (0.4 x L x W2) was calculated, values shown are the mean of five tumors (mm^3); bars; SD. (B) Tumor xenografts 28 days after treatment. (C) Representative photomicrograph showing the presence of apoptotic bodies detected by the TUNEL method in a tumor sample from a mouse 28 days after treatment with Wr-T and p16 peptide mixture (right), note the absence of apoptotic cells in an untreated sample (left).

(CDKNA2A, $p16^{INK4a}$) expression only in 6 of 532 patients with clear cell renal cell carcinoma (30). In Japan, Kawada *et al* reported that only 7 of 91 patients did not show $p16^{INK4a}$ expression (31). These findings suggested racial differences. However, our results were contrary to those obtained by Kawada *et al* (data not shown). Specifically, we studied the expression of cyclin inhibitors in renal cell carcinoma cell lines and patients with this disease using immunostaining, and found that none of the cell lines showed $p16^{INK4a}$ expression by Western blotting and that some of the cell lines did not show $p27^{Kipl}$ expression, either; in addition, cell lines from only 2 of 84 renal cell carcinomas stained positive. During our study, phosphorylated Rb was detected in all renal cell carcinoma cell lines studied (Fig. 1). This finding suggests dysfunction of cyclin inhibitors and thus the possibility that the growth of renal cell carcinoma cells may be inhibited if the function of cyclin inhibitors is restored. Presuming that many Japanese patients with renal cell carcinoma show no $p16^{INK4a}$ expression, we thought it useful to establish a new cancer therapy targeting $p16^{INK4a}$ in the present study.

We therefore synthesized the amino acid sequence representing the minimal function of $p16^{INK4a}$, and delivered this sequence to renal cell carcinoma cell lines using an intracellular peptide/protein delivery system, which had already been established by our group. As a result, cell growth was almost completely inhibited in 7 of the 10 cell lines (Fig. 2). The reason for the poor response in the remaining 3 cell lines is still unknown, but there seems to be another cell-growth-regulating system. Although the $p16^{INK4a}$ peptide, as well as the PTD of our peptide/protein delivery system, has a sequence consisting of 9 arginines, $p16^{INK4a}$ used as a complex with the peptide/protein delivery system was found to be more effective than $p16^{INK4a}$ used alone. Different effects were shown when the same PTD was used. This finding suggests that the efficiency of peptide delivery is affected not only by the PTD itself but also by the sequence of the peptides-binding site. It seems that $p16^{INK4a}$ impairs the function of the Cyclin D-CDK4/6 complex and thus inhibits Rb phosphorylation, because this complex induces Rb phosphorylation. In cells transduced with the $p16^{INK4a}$ peptide, Rb phosphorylation is inhibited and the delivered peptide seems to function in place of the normal $p16^{INK4a}$ molecule (Fig. 4). In our previous report on B cell lymphoma, cells with the peptide showed G1 arrest and induction of apoptosis. In addition, the number of renal cell carcinoma cells in the G2/M phase obviously decreased and that of cells in the G1/G0 phase increased, suggesting G1 arrest in renal cell carcinoma cells (Fig. 3). Moreover, apoptosis seemed to have been induced in such cells, because Annexin V-positive cells increased over time, with no cells negative for Annexin V staining and positive only for PI (Fig. 5), and because of the increase of apoptotic bodies in the transplanted tumors treated with the peptides as demonstrated by the TUNEL assay (Fig. 6C). These findings indicated that peptide delivery not only inhibited cell growth but also induced tumor cell apoptosis.

Since the *in vitro* experiment suggested the feasibility of treatment of renal cell carcinoma through peptide delivery, we experimentally treated tumors using a nude mouse model of transplanted human tumor. During the previously reported experiment in B cell lymphoma, peptides were directly inoculated as a single dose into the tissue surrounding a tumor formed by transplantation. Tumor shrinkage was then noted, but regrowth of the tumor was detected from day 5 after peptide inoculation (21). This finding suggested short-term stability of peptides after inoculation and thus a limited effect of treatment with single-dose peptide inoculation. In the present experimental treatment of renal cell carcinoma, we therefore compared a single-dose regimen with a regimen consisting of 3 doses administered at 1-week intervals. The single-dose regimen clearly provided a greater therapeutic effect than the control regimen, while the 3-dose regimen provided much greater therapeutic effect; tumors had completely disappeared 28 days after the initiation of the first treatment (Fig. 6A and B). This finding indicates that renal cell carcinoma can be treated with the $p16^{INK4a}$ peptide and that peptide therapy requires more than one dose.

The $p16^{INK4a}$ functional peptide and transporter peptide administered during the present study are not cell-specific. Since this complex is also delivered to normal cells, its toxicity becomes an issue. In a study conducted in patients with B cell lymphoma, peptide delivery was noted in normal lymphocytes, but induction of apoptosis was not (21). In addition, in experimental treatment of human glioma in mice with transplanted human glioma, the peptide was systemically administered via the heart, as was done in the present study (22). As a result, tumor shrinkage was noted, but no pathological abnormalities were detected in the normal cerebral tissue surrounding the transplanted tumor or in other normal organs. During the present experimental treatment in mice with transplanted human renal cell carcinoma, normal tissues (kidney, liver and spleen) were histologically examined. No significant differences or abnormalities were noted between the treated and untreated groups, and no abnormalities were seen. In the experiment performed to assess the effect on cell growth, cell growth was slightly inhibited by the use of Wr-T alone, but the observational experiment did not reveal any apoptosis. These findings suggest that Wr-T delays the cell cycle, with no cell-killing effect.

The present findings indicate the efficacy of our therapy, in which a functional peptide is delivered using the transporter developed by our research group, for renal cell carcinoma. This therapy, which seems to have little effect on normal cells, will pave the way for clinical studies.

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