Systemic transduction of p16^{INK4A} antitumor peptide inhibits the growth of MBT-2 mouse bladder tumor cell line grafts

TORU SHIMAZUI¹, KAZUHIRO YOSHIKAWA², JUN MIYAZAKI³, TAKAHIRO KOJIMA³, HIROMU INAI⁴, SATOSHI ANDO³, HIROTSUGU UEMURA⁵, KAZUHIKO UCHIDA⁶ and HIROYUKI NISHIYAMA³

¹Department of Urology, Ibaraki Clinical Education and Training Center, Faculty of Medicine, University of Tsukuba, Tsukuba; ²Division of Clinical Research Promotion, Aichi Medical University, Nagakute; ³Department of Urology, Faculty of Medicine, University of Tsukuba, Tsukuba; ⁴Department of Urology, International University of Health and Welfare, Otawara; ⁵Department of Urology, Kinki University, Higashiosaka; ⁶Department of Molecular Oncology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

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Abstract. p16^{INK4A} (p16), a key molecule in bladder tumor development, inhibits the activities of cyclin-dependent kinases (CDKs) and maintains the retinoblastoma protein (pRb) in its active hypophosphorylated state. Following the finding that the p16 antitumor peptide dramatically inhibits the growth of aggressive leukemia/lymphoma through the restoration of p16 function using the Wr-T peptide transporter system, in this study, we developed a systemic therapy using mouse-p16 peptide (m-p16) in subcutaneous p16-null mouse bladder tumors. In vitro analysis showed that the growth of p16-null bladder tumor cells and the hyperphosphorylation of their pRbs were inhibited by p16 transduction in a concentration-dependent manner. In an animal model, p16-null MBT-2 cells were injected subcutaneously into KSN/SKC nude mice. The systemic delivery of the m-p16 peptide using Wr-T by cardiac injection significantly inhibited the growth of solid MBT-2 tumors compared with the control phosphate-buffered saline (PBS) injection. Histological examination by TUNEL staining revealed that apoptosis was increased and pRb phosphorylation was inhibited. Thus, the systemic peptide delivery of p16 restores the hypophosphorylation of pRb and may be a useful tool for the treatment of bladder tumors.

Introduction

Human urothelial carcinoma (UC) evolves via the accumulation of numerous genetic alterations, with the loss of p53 and p16^{INK4A} (p16) functions representing important stages in the development of superficial lesions and their progression to malignant disease (1). p16 inhibits the activities of cyclin-dependent kinases (CDKs), which maintain the retinoblastoma protein (pRb) in its active hypophosphorylated state (2). p16 gene transfection has been shown to result in a marked decrease in pRb phosphorylation, a decrease in cell proliferation and the suppression of the tumorigenicity of bladder tumor cell lines (3, 4). It has been reported that the p16 antitumor peptide dramatically inhibits the growth of highly aggressive leukemia/lymphoma through the restoration of p16 function (5).

Current standard drug therapies involve the intravesical instillation of a chemotherapeutic agent or bacillus Calmette-Guérin (BCG) for non-muscle-invasive bladder tumors (6) and cisplatin-based systemic chemotherapy for locally advanced and metastatic bladder tumors. BCG instillation may cause severe adverse events, such as hematuria, fever, irritation or contraction and resistance to BCG remains an unsolved issue. By contrast, in metastatic tumors, the treatment of chemorefractory cases and maintenance therapy for chemosensitive cases are important issues in the development of a new therapeutic approach, which may be a different procedure from chemotherapeutic agents.

We hypothesized that a minimum inhibitory sequence (MIS) peptide of p16 (p16-MIS) may play a role in the anti-tumor effect in p16-deficient bladder tumor cell lines using the Wr-T system. The aim of this study was to assess the effect of the transfer of the p16 peptide on several bladder tumor cell lines in vitro and the effect of the mouse MIS peptide of p16 (m-p16-MIS) on a mouse bladder tumor cell line (MBT-2) in vivo.

Materials and methods

Cell lines. The human bladder tumor cell lines, 253J, 575A, J82, Jon, RT112, RT4, SW1710, SW800, T24 and VMCB1 (kindly provided by Dr Schalken, Radboud Medical Center, Nijmegen, The Netherlands), the mouse bladder tumor cell line, MBT-2, and the mouse kidney tumor cell line, Renca, were cultured in RPMI-1640 containing 10% inactivated fetal bovine serum.
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Table I. PCR primers and conditions.

<table>
<thead>
<tr>
<th>Molecule fragment</th>
<th>Sequence F:</th>
<th>Annealing temperature (˚C)</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>p16</td>
<td>ATAGTTACGGTGCGGAGGCC</td>
<td>60</td>
<td>536</td>
</tr>
<tr>
<td></td>
<td>TGGTTACGTCCCTCTGGTG</td>
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</tr>
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<td>Cyclin D</td>
<td>AAAGACATTGTTGGATATCTTTT</td>
<td>55</td>
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<td>CGGAGATGGTTGATACCG</td>
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<td>CTCTGCTACGAGGGGCC</td>
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<td>110</td>
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<td>TGGAAGGGGAAATGTCATTTA</td>
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<tr>
<td></td>
<td>CTCCTTAATGCACGCAGATTTC</td>
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F, forward; R, reverse.

(IBM; Gunma, Japan), at 37˚C under an atmosphere of 5% CO₂. After the transfer of peptides to the cells, the viability of the cell line was evaluated using a WST-8 Cell Counting kit-8 (#347-07621 Dojindo, Kamimashiki, Japan).

Peptide synthesis. All peptides including Wr-T and r9-p16-MIS for human and mouse 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 KETWWETWWTEWWTEWSQGPGrrrrrrrr (r, D-enantiomer arginine) (5,7). For synthesis of the respective p16-MIS for human and mouse, the 10-amino acid sequence FLDTLVVLHR and FLDTLVVLHG, identified as the MIS of p16 by Fåhraeus et al (8), 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 into 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 peptide were mixed in 10 µl of distilled water at room temperature for 60 min (final concentrations, 5 µmol/l Wr-T and 8 µmol/l r9-p16-MIS). The solution was then added directly to 190 µl of RPMI-1640 containing 5% fetal bovine serum to obtain the final concentration indicated. In vivo peptide delivery to the solid bladder tumor was performed by injecting the Wr-T/r9-p16-MIS peptide mix (50 nmol Wr-T, 80 nmol r9-p16-MIS) into the hearts of mice bearing tumors that had grown to a diameter of 3 mm (tumor volume, ~15 mm³). The control groups were administered 100 µl of phosphate-buffered saline (PBS) without peptide, Wr-T, or p16 peptide alone dissolved in 100 µl of PBS and injected as previously described (7).

Reverse transcription-PCR. Total RNA (5 µg) was extracted from each tumor cell line using TRIZol reagent (#15596-026, Life Technologies, Tokyo, Japan). Briefly, cells were washed with PBS and lysed with 1 ml of TRIZol reagent. Chloroform (200 µl) was added and the mixture was centrifuged at 4˚C and 12,000 x g for 15 min. The liquid phase was precipitated with isopropanol. The RNA pellets were dissolved in Tris-EDTA (TE) buffer. Subsequently, cDNA was synthesized from the extracted RNA using random primers and a cDNA synthesis kit (#4368814 High Capacity cDNA Transcription kits; Applied Biosystems, Tokyo, Japan). Reverse transcription-PCR was then carried out with a Taq PCR Core kit (#201225; Qiagen, Tokyo, Japan). Amplification conditions and primer sequences are listed in Table I. The sense/antisense primer sequences for CDK4, CDK6 and cyclin D were also described in a previous publication (9).

Western blot analysis. Cells were promptly lysed with SDS sample lysis buffer and the extracts were separated by SDS-PAGE using 12.5 to 15% Bis-Tris gradient gels (SuperSepAce, Wako, Osaka, Japan). Proteins were transferred onto a Hybond-P membrane (#RPN2020F GE Healthcare Japan, Tokyo, Japan), blocked with 5% dried milk and 1% normal goat serum-PBS and then sequentially probed with mouse monoclonal anti-p16INK4 antibody (Clone: F-12, #SC1661; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse monoclonal anti-p21WAF1 antibody (Clone: EA10; Oncogene Research Products, Boston, MA, USA), mouse monoclonal anti-p27Kip1 antibody (Clone: 1B4; Novocastra Laboratories Ltd, Newcastle, UK), mouse monoclonal anti-pRb antibody (Clone: LM95.1; Oncogene Research Products), rabbit polyclonal anti-phospho-Ser780 pRb antibody (#9307S; Cell Signaling Technology Japan, K.K., Tokyo, Japan), mouse monoclonal anti-actin antibody (Clone: AC-74; Sigma, St. Louis, MO, USA). Immune complexes were visualized with ECL Western Blotting Detection Reagents (#RP2109; GE Healthcare, Tokyo, Japan) according to the manufacturer’s instructions and signals were visualized and digitally captured using an image analyzer (LAS 4000; GE Healthcare).
Mouse tumor models. Four-week-old female KSN nude mice were obtained from SLC, Inc. (Hamamatsu, Japan). A total of 100 µl of a PBS suspension containing 2.0x10^6 cells of the mouse bladder tumor cell line, MBT-2, was injected subcutaneously (s.c.) into the flanks of each mouse to form a solid tumor nodule. Animal experiments performed in this study were approved by the Laboratory Animal Resource Center, University of Tsukuba, Tsukuba, Japan. All mouse procedures, euthanasia and surgery, including bladder tumor transplantations and peptide injections, were carried out painlessly or under anesthesia within the strict guidelines of the Laboratory Animal Resource Center, University of Tsukuba.

Immunohistochemical and TUNEL staining. Tumors and other organs were fixed in 10% neutral-buffered formalin overnight and were then processed, paraffin-embedded, sectioned, mounted onto slides and stained by the standard hematoxylin and eosin method. Serial paraffin sections (4 µm thick) were stained using rabbit polyclonal anti-phospho-Ser780 pRb antibody (diluted 1:100, #9307S; Cell Signaling Technology), followed by the universal immuno-enzyme polymer method (#714342 N‑Histofine Simple Stain Mouse MAX PO; Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's instructions. The sections were developed with 3,3′-diaminobenzidine tetrahydrochloride, containing 0.03% hydrogen peroxide, and counterstained with hematoxylin. Apoptotic cells were detected in tumors harvested from mice 48 h after peptide administration. Apoptosis in the tumor sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay with the use of an ApopTag Peroxidase In Situ Apoptosis Detection kit (#S7100; Nippon Chemicon, Tokyo, Japan) following the manufacturer's instructions.

Results

Expression of p16, phosphorylated pRb and related molecules in human bladder tumor cell lines. Out of the 10 human bladder tumor cell lines, 7 lines did not express p16 with pRb phosphorylation (Fig. 1) on the mRNA and protein levels. p21 and p27, which are downstream of p53, are expressed on a protein level in most p16-deficient lines. The mRNA expression of CDK4 and CDK6, which are further downstream of p16, was observed in all the 10 lines (Fig. 2).

Growth inhibition of bladder tumor cells after r9-p16-MIS transduction assessed by cell proliferation assay. We then suppressed the growth of the bladder tumor cells using the Wr-T-transported r9-p16-MIS. The r9-p16-MIS was introduced into each bladder tumor cell line beginning with 1.0x10^5 cells per incubation with Wr-T (final concentrations, 5 µmol/l Wr-T and 2-8 µmol/l r9-p16-MIS) (Fig. 3) and cell growth was monitored by the WST-8 cell proliferation assay. The administration of r9-p16-MIS produced some growth suppression in 7 out of the 10 cell lines. In 4 cell lines, >50% inhibition was observed at concentrations of at least 8 µmol/l of r9-p16-MIS, e.g., 80, 50, 50 and 95% in the 253J, 575A, RT4 and SW1710 cells, respectively (Fig. 4). These cell lines demonstrated a significant decrease in the expression of phosphorylated pRb following r9-p16-MIS transduction (Fig. 5).

In vivo bladder tumor suppression by Wr-T and r9-p16 transduction system. In view of the therapeutic potential of the Wr-T and r9-p16-MIS delivery system, we investigated the efficacy of this system for the treatment of the mouse bladder tumor cell line, MBT-2, using allografts transplanted s.c. into KSN nude mice. The expression of p16 was not observed in the MBT-2 cells (Fig. 6A) and that of phosphorylated pRb was downregulated.
following mouse r9-p16-MIS delivery in a concentration-dependent manner (Fig. 6B). When the tumors had reached 3 mm in diameter, we administered the Wr-T and mouse r9-p16-MIS mixture to the mice via cardiac delivery. A significant decrease in tumor size was observed following Wr-T and mouse r9-p16-MIS transduction compared with the peptide-free tumors (PBS vs. single and triple doses of Wr-T and mouse r9-p16-MIS, p=0.011 and 0.00034, respectively) (Fig. 7). By the 5th day after peptide transduction, the diameter of the peptide-free tumor was 33% larger than that of the tumors treated with Wr-T and mouse r9-p16-MIS.

At 72 h post-transduction, the expression of phospho-Ser780 pRb in the tumors treated with Wr-T and mouse r9-p16-MIS mixture was decreased, as assessed by immunohistochemistry (Fig. 8). By contrast, TUNEL analysis showed an increase in the presence of positively-stained apoptotic bodies in the tumors treated with the Wr-T and mouse r9-p16-MIS mixture (Fig. 8).

Discussion

The p16 gene is located on the short arm of chromosome 9 at the p21 locus. p16 is a kinase inhibitor that inhibits the activity of CDK4 and CDK6 to phosphorylate pRb. In bladder tumors, the abnormality of chromosome 9 has been reported as an early genetic event in both non-muscle- and muscle-invasive UC (10). Thus, the p16-pRb pathway could be considered a therapeutic target based on the molecular mechanism of bladder tumor carcinogenesis.

During investigations of the introduction of the p16 gene into cell lines, growth arrest and suppression of tumorigenicity were observed in ovarian (11) and bladder (3) cancers. We hypothesized that peptide transfer is more acceptable than gene transfection for clinical application; therefore, we designed a protocol for systemic and local administration to inhibit the growth of transplanted and orthotopic bladder tumors. We synthesized the p16-MIS amino acid sequence, which represented the minimal function of p16, and delivered it into bladder tumor cell lines using a peptide delivery system as described by Kondo et al (5). As shown in Fig. 7, an in vitro analysis demonstrated that p16-MIS inhibited tumor growth; this effect was not only dependent on the loss of p16 but also on the degree of pRb phosphorylation.

In the present study, the overexpression of p16 was observed in some of the bladder tumor cell lines irrespective of the pRb phosphorylation status. As shown in Fig. 1, p16 was overexpressed rather than normally expressed in some of the cell lines, e.g., 575A. Recently, Nakazawa et al (12) reported that p16 overexpression was observed in 16 to 50% of cytology samples of bladder tumors. A possible mechanism of p16 overexpression was explained as the self-regulation that accompanies abnormally high levels of cell proliferation. Alternatively, whether or not the expression of p16 is functional should be clarified. Asamoto et al (13) suggested that the overexpression of p16 mRNA indicates the deregulation of pathways involving the p16 gene in mice. Since p16-MIS transduction is most likely useful to the p16-expressing 575A bladder tumor cells when accompanying phosphorylated pRb accumulation, another explanation may be that the p16 protein in these cell lines may not function properly through the p16-pRb molecular pathway.

Due to the delivery of p16-MIS, pRb phosphorylation was shown to be reduced by western blot analysis, irrespective of the previous expression/phosphorylation of pRb, as indicated in Fig. 5. Our histological analysis of tissues post-p16 delivery showed that pRb phosphorylation was inhibited and that the delivery peptide seemed to function in place of the normal p16 molecule. In addition, apoptosis was deemed to be induced after the p16-MIS transfer as apoptotic bodies were increased in the transplanted tumors treated with the p16 peptide, as shown by TUNEL staining. These findings demonstrated that the present peptide delivery system induced tumor cell apoptosis as well as cell growth inhibition.

A comparison of a single dose with 3 doses indicated that multiple administrations were more effective in suppressing tumor growth. However, tumors treated with 3 doses were still histologically active, suggesting that this delivery system is limited. Since muscle-invasive or metastatic UC frequently contains several genetic alterations, including p53 abnormality (14), a combination of either the transduction of another...
Figure 6. The mouse bladder tumor cell line, MBT-2, does not express p16 on either the mRNA or protein levels. (A) Renca, a mouse kidney tumor cell line, was used as the positive control. (B) The phosphorylation of Rb in MBT-2 cells was decreased following mouse p16-MIS peptide transduction in concentration-dependent manner.

Figure 7. (A) The mouse tumor model demonstrates that the cardiac injection of mouse p16-MIS with Wr-T inhibited the MBT-2 allografts as compared with the control PBS injection. (B) The growth curves indicate that p16 with Wr-T significantly inhibited tumor growth by 1 (P=0.0110) or 3 injections (p=0.00034).

Figure 8. Immunohistochemical staining of the MBT-2 allografts shows that the expression of phospho-Ser780 pRb was decreased at 72 h after p16 peptide transduction with Wr-T. At the same time, TUNEL staining reveals that apoptosis was increased following p16 transduction.
peptide, e.g., p53, or chemotherapeutic agents may be more effective for the systemic treatment of metastatic UC.

Systemic administration, as shown in the animal model, demonstrated that a p16-MIS transfer system may be available for clinical use in patients with systemic disease, such as distant metastases of UC. Current standard systemic chemotherapy for metastatic UC is a combination of gemcitabine and cisplatin, i.e., GC therapy (15). Although GC therapy is expected to produce a 60-70% response rate, refractory cases or severe adverse events are often experienced. Thus, patients with refractory tumors or severe adverse events may be potential candidates for p16-MIS transduction therapy. Sequential and maintenance therapy using p16-MIS may be a treatment option for a well-controlled case following systemic chemotherapy.

A limitation of this study was that we were unable to evaluate the efficacy of the p16 peptide against non-muscle-invasive bladder tumors by intravesical instillation. Intravesical BCG instillation is the current standard treatment for moderate-to-high-risk UC of the bladder. Low-grade non-muscle-invasive UC has a limited gene alteration and the p53 mutation is not very frequent (14). The findings described above support the application of the intravesical instillation of a p16 peptide to prevent the implantation of floating tumor cells after transurethral resection, since its instillation is similar to an in vitro peptide transfer. Of note, Sato et al (16) reported that the overexpression and phosphorylation of pRb in bladder tumor cells predicts a poor response to BCG therapy. Thus, combined therapy by p16 peptide transfer with BCG instillation may be a promising treatment for non-muscle-invasive bladder tumors, since restoring pRb function by p16 peptide transduction may be effective in treating BCG-refractory bladder tumors. The following step after the animal model is the application of p16-MIS delivery alone or in combination with BCG instillation using a mouse intravesical implantation model of the MBT-2 cell line (17) to examine the toxicity of p16 peptide transfer to normal organs, particularly to normal urothelial cells of urinary bladder mucosa.

In conclusion, the delivery of p16-MIS using a novel peptide transduction system may be a new therapeutic option for metastatic UC; however, additional experiments are required to investigate the efficacy of the local administration and toxicity of the p16 peptide.

Acknowledgements

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References