effective for lung metastasis of renal cell carcinoma. A multicenter open study. *Int J Clin Oncol* 2006;11:434–40.
20. Akaza H, Kawai K, Tsukamoto T, Fujioka T, Tomita Y, Kitamura T, et al. Successful outcomes using combination therapy of interleukin-2 and interferon-α for renal cell carcinoma patients with lung metastasis. *Jpn J Clin Oncol* 2010;40:684–9.
21. Mizumori O, Zembutsu H, Kato H, Tsunoda T, Miya F, Morizono T, et al. Identification of a set of genes associated with response to interleukin-2 and interferon-α combination therapy for renal cell carcinoma through genome-wide gene expression profiling. *Exp Therapeu Med* 2010;1:955–61.
22. Japanese Urological Association and Japanese Society of Pathology. Response criteria for urological cancer treatment. *Jpn J Urol* 1992;83:447–72.
23. Miller AB, Hoogstraten B, Straquet M, Winkler A. Reporting results of cancer treatment. *Cancer* 1981;47:207–14.
24. Gore ME, Griffin CL, Hancock B, Patel PM, Aitchison M, James N, et al. Interferon alfa-2a versus combination therapy with interferon alfa-2a, interleukin-2, and fluorouracil in patients with untreated metastatic renal cell carcinoma (MRC RE04/EORTC GU 30012): an open-label randomized trial. *Lancet* 2010;375:641–8.
25. Zini L, Capitanio U, Perrotte P, Jeldres C, Shariat SF, Arjane P, et al. Population-based assessment of survival after cytoreductive nephrectomy versus no surgery in patients with metastatic renal cell carcinoma. *Urology* 2009;73:342–6.
26. Flanigan RC, Salmon SE, Blumenstein BA, Bearman SI, Roy V, McGrath PC, et al. Nephrectomy followed by interferon alfa-2b compared with interferon alfa-2b alone for metastatic renal-cell cancer. *N Engl J Med* 2001;345:1655–9.
27. Stafford HS, Saltzstein SL, Shimasaki S, Sanders C, Downs TM, Robins Sadler G. Racial/ethnic and gender disparities in renal cell carcinoma incidence and survival. *J Urol* 2008;179:1704–8.
28. Jeppesen AN, Jensen HK, Donskov F, Marcussen N, von der Maase H. Hyponatremia as a prognostic and predictive factor in metastatic renal cell carcinoma. *Br J Cancer* 2010;102:867–72.
29. Figlin R, Gitlitz B, Franklin J, Dorey F, Moldawer N, Rausch J, et al. Interleukin-2-based immunotherapy for the treatment of metastatic renal cell carcinoma: an analysis of 203 consecutively treated patients. *Cancer J Sci Am* 1997;3:S92–7.
30. Fumagalli LA, Vinke J, Hoff W, Ypma E, Brivio F, Nespoli A. Lymphocyte counts independently predict overall survival in advanced cancer patients: a biomarker for IL-2 immunotherapy. *J Immunother* 2003;26:394–402.
31. Pace L, Vitale S, Dettori B, Palombi C, La Sorsa V, Belardelli F, et al. APC activation by IFN-α decrease regulatory T cell and enhances Th cell functions. *J Immunol* 2010;184:5069–79.
32. Golding A, Rosen A, Petri M, Akhter E, Andrade F. Interferon-alpha regulates the dynamic balance between human activated regulatory and effector T cells: implications for antiviral and autoimmune responses. *Immunology* 2010;131:107–17.
33. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 2010;33:153–65.
34. Tatsugami K, Eto M, Naito S. Influence of immunotherapy with interferon-α on regulatory T cells in renal cell carcinoma patients. *J Interferon Cytokine Res* 2010;30:43–7.
35. Motzer RJ, Hutson TE, Tomczak P, Michaelson D, Bukowski RM, Rixe O, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med* 2007;356:115–24.
36. Motzer RJ, Michaelson MD, Rosenberg J, Bukowski RM, Curti BD, George DJ, et al. Sunitinib efficacy against advanced renal cell carcinoma. *J Urol* 2007;178:1883–7.
37. Tomita Y, Shinohara N, Yuasa T, Fujimoto H, Niwakawa M, Mugiya S, et al. Overall survival and updated results from a Phase II study of sunitinib in Japanese patients with metastatic renal cell carcinoma. *Jpn J Clin Oncol* 2010;40:1166–72.
38. Negrier S, Jäger E, Porta C. Efficacy and safety of sorafenib in patients with advanced renal cell carcinoma with and without prior cytokine therapy, a subanalysis of TARGET. *Med Oncol* 2010;27:899–906.

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

KENJI ZENNAMI[1], KAZUHIRO YOSHIKAWA[1], EISAKU KONDO[4], KOGENTA NAKAMURA[1], YOSHIAKI YAMADA[1], MARCO A. DE VELASCO[3], MOTOYOSHI TANAKA[3], HIROTSUGU UEMURA[3], TORU SHIMAZUI[4], HIDEYUKI AKAZA[5], SHINSUKE SAGA[6], RYUZO UEDA[7] and NOBUAKI HONDA[1]

[1]Department of Urology, Aichi Medical University School of Medicine, Nagakute, 480-1195 Aichi; [2]Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, 464-8681 Aichi; [3]Department of Urology, Kinki University School of Medicine, Osaka-Sayama, 589-8511 Osaka; [4]Department of Urology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, 305-8575 Ibaraki; [5]Department of Strategic Investigation on Comprehensive Cancer Network Research Center for advanced Science and Technology, The University of Tokyo, Meguro-ku, 153-8904 Tokyo; [5]Department of Pathology, Aichi Medical University School of Medicine and [6]Nagoya City University Graduate School of Medical Sciences, Nagoya, 467-8601 Aichi, Japan

Received February 4, 2011; Accepted March 8, 2011

DOI: 10.3892/or.2011.1290

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.

Correspondence to: Dr Kazuhiro Yoshikawa, Division of Clinical Research Promotion, Aichi Medical University, Nagakute, Aichi 480-1195, Japan
E-mail: yoshikaw@aichi-med-u.ac.jp

Key words: PTD, p16, renal cell carcinoma

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

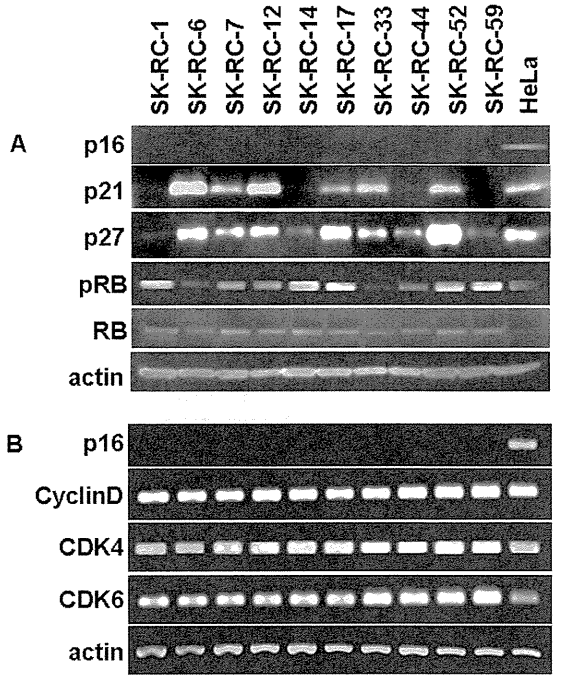


Figure 1. Loss of $p16^{INK4a}$ expression was correlated to cell cycle pathway genes in 10 human renal cell carcinoma cell lines by Western blotting and RT-PCR. (A) Endogenous protein expression of $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$ RB and phospho-Ser^{780} pRB in the examined cell lines by immunoblotting. (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.

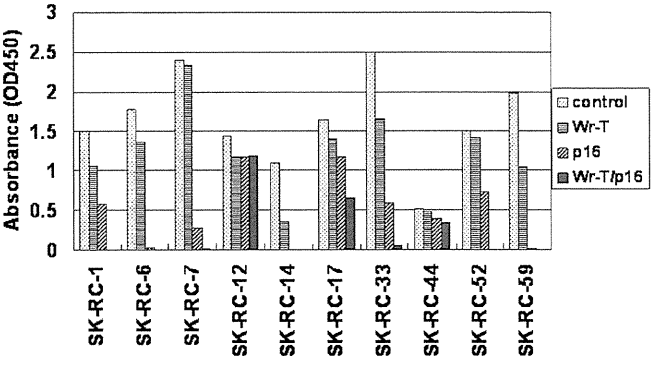


Figure 2. Cell growth suppression by transduction of Wr-T and p16 peptides. Ten representative cases of renal cell carcinoma cell lines are shown. Proliferation of cells treated with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 8 μmol/l) was compared with cells treated without peptides,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

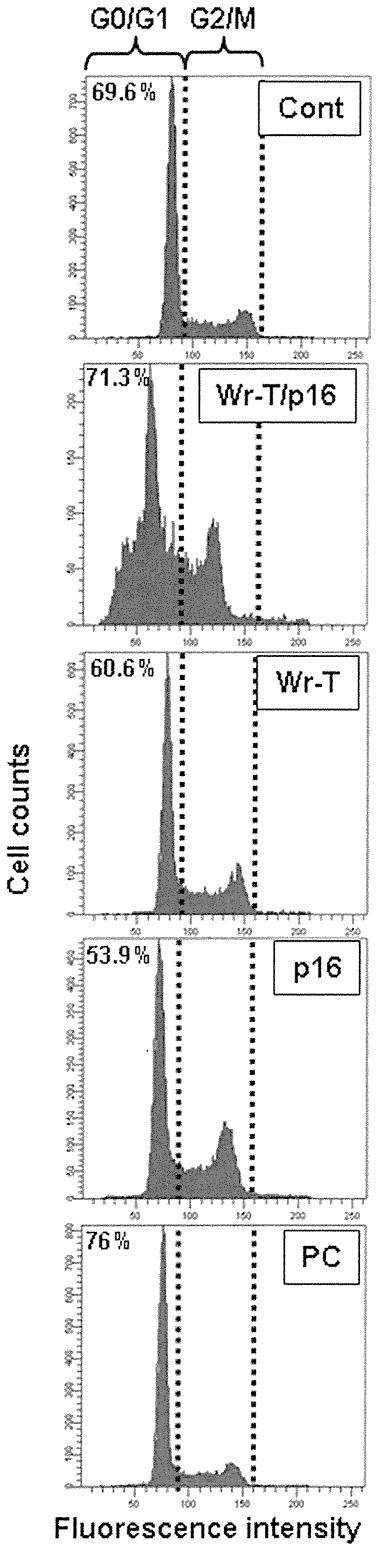


Figure 3. Enlarged G0-G1 phase in renal cell carcinoma cells treated with Wr-T and p16 peptides mixture. Cell cycle profiles of propidium iodide stained SK-RC-7 cells transduced with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 6 μmol/l), without peptides, with Wr-T (5 μmol/l) alone, and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

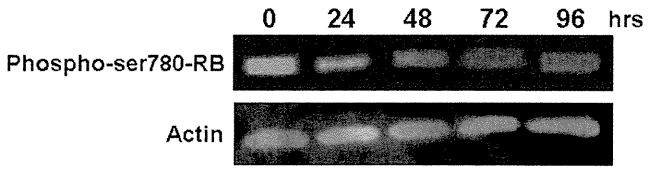


Figure 4. Reduced expression of phospho-Ser^{780} pRB in Wr-T/r9-p16 MIS-introduced cells assessed by immunoblotting (Wr-T, 5 μmol/l; p16, 8 μmol/l).

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^{Kip1} (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

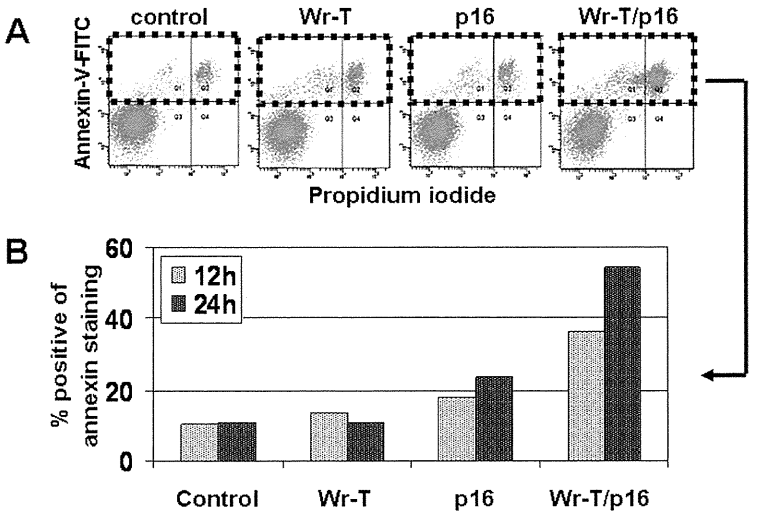


Figure 5. Apoptotic induction of renal cell carcinoma cells treated with Wr-T and p16 peptides mixture. The SK-RC-7 cells were treated with the Wr-T and p16 peptides mixture (Wr-T, 5 μmol/l; p16 MIS, 8 μmol/l), without peptides, with Wr-T (5 μmol/l) alone, and with p16 (8 μmol/l) alone. (A) Flow cytometry 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).

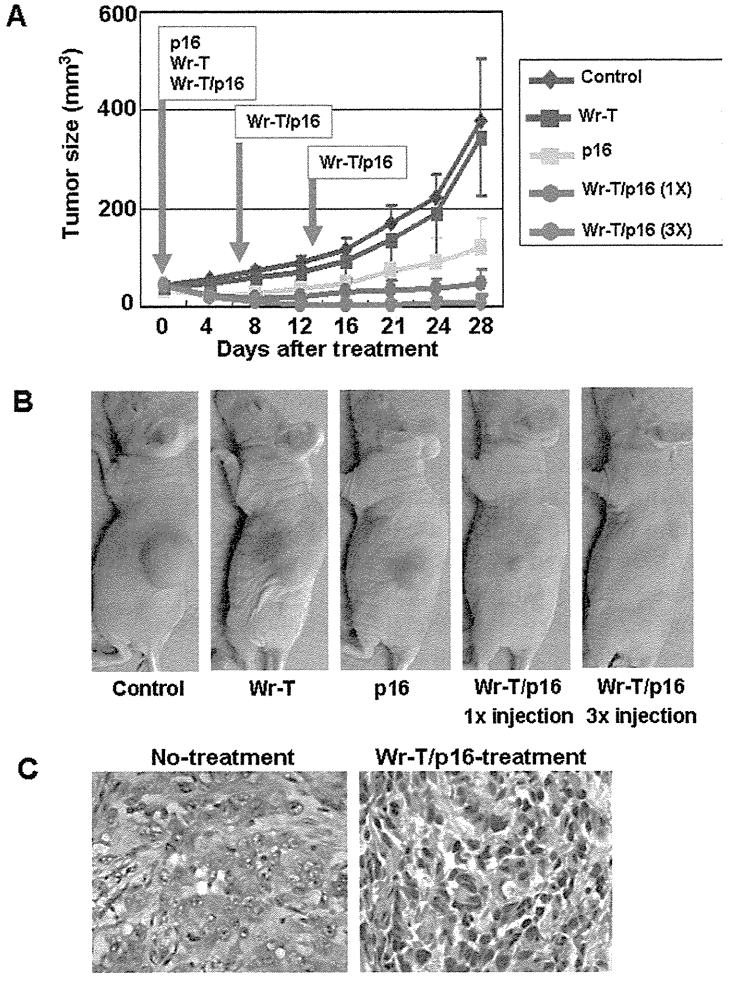


Figure 6. Effects of *in vivo* administration of Wr-T and p16 peptides mixture on tumor growth. SK-RC-7 cells ($1x10^6$) were transplanted into the backs of the nude mice. Treatments were performed as described in Materials and methods using 5 mice in each group. (A) Growth curves of tumors. Sizes of 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 $p27^{Kip1}$ 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.

Acknowledgements

We are grateful to Mr. Makoto Naruse in Division of Advanced Research Promotion, Aichi Medical University for his technical assistance with FACSCanto data acquisition, and Ms. Shino Kojima and Ms. Norika Yamada in Department of Pathology, Aichi Medical University for their technical assistance.

References

1. Wirth MP: Immunotherapy for metastatic renal cell carcinoma. Urol Clin North Am 20: 283-295, 1993.
2. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR and Louie AC: Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. J Clin Oncol 13: 688-696, 1995.
3. Medical Research Council Renal Cancer Collaborators: Interferon-alpha and survival in metastatic renal carcinoma: early results of a randomised controlled trial. Lancet 353: 14-17, 1999.

4. Motzer RJ, Bacik J, Murphy BA, Russo P and Mazumdar M: Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J Clin Oncol 20: 289-296, 2002.
5. Akaza H, Tsukamoto T, Onishi T, Miki T, Kinouchi T and Naito S: A low-dose combination therapy of interleukin-2 and interferon-alpha is effective for lung metastasis of renal cell carcinoma: a multicenter open study. Int J Clin Oncol 11: 434-440, 2006.
6. Klapper JA, Downey SG, Smith FO, Yang JC, Hughes MS, Kammula US, Sherry RM, Royal RE, Steinberg SM and Rosenberg S: High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma: a retrospective analysis of response and survival in patients treated in the surgery branch at the National Cancer Institute between 1986 and 2006. Cancer 113: 293-301, 2008.
7. Gollob JA, Rathmell WK, Richmond TM, Marino CB, Miller EK, Grigson G, Watkins C, Gu L, Peterson BL and Wright JJ: Phase II trial of sorafenib plus interferon alfa-2b as first- or second-line therapy in patients with metastatic renal cell cancer. J Clin Oncol 25: 3288-3295, 2007.
8. Ryan CW, Goldman BH, Lara PN Jr, Mack PC, Beer TM, Tangen CM, Lemmon D, Pan CX, Drabkin HA and Crawford ED; Southwest Oncology Group: Sorafenib with interferon alfa-2b as first-line treatment of advanced renal carcinoma: a phase II study of the Southwest Oncology Group. J Clin Oncol 25: 3296-3301, 2007.
9. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM and Figlin RA: Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356: 115-124, 2007.
10. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D and Ravaud A; RECORD-1 Study Group: Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372: 449-456, 2008.
11. Escudier B, Szczylik C, Hutson TE, Demkow T, Staehler M, Rolland F, Negrier S, Laferriere N, Scheuring UJ, Cella D, Shah S, Bukowski RM: Randomized phase II trial of first-line treatment with sorafenib versus interferon Alfa-2a in patients with metastatic renal cell carcinoma. J Clin Oncol 27: 1280-1289, 2009.
12. Motzer RJ, Hudes GR, Curti BD, McDermott DF, Escudier BJ, Negrier S, Duclos B, Moore L, O'Toole T, Boni JP and Dutcher JP: Phase I/II trial of temsirolimus combined with interferon alfa for advanced renal cell carcinoma. J Clin Oncol 25: 3958-3964, 2007.
13. Green M and Loewenstein PM: Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell 55: 1179-1188, 1988.
14. Frankel AD and Pabo CO: Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55: 1189-1193, 1988.
15. Schwarze SR, Ho A, Vocero-Akbani A and Dowdy SF: In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285: 1569-1572, 1999.
16. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA and Dowdy SF: Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med 12: 1449-1452, 1998.
17. Derossi D, Joliot AH, Chassaing G and Prochiantz A: The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269: 10444-104450, 1994.
18. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L and Rothbard JB: The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci USA 97: 13003-13008, 2000.
19. Fisher PM, Krausz E and Lane DP: Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation. Bioconjug Chem 12: 825-841, 2001.
20. Joliot A and Prochiantz A: Transduction peptides: from technology to physiology. Nat Cell Biol 6: 189-196, 2004.
21. Kondo E, Seto M, Yoshikawa K and Yoshino T: Highly efficient delivery of p16 antitumor peptide into aggressive leukemia/lymphoma cells using a novel transporter system. Mol Cancer Ther 3: 1623-1630, 2004.
22. Kondo E, Tanaka T, Miyake T, Ichikawa T, Hirai M, Adachi M, Yoshikawa K, Ichimura K, Ohara N, Moriwaki A, Date I, Ueda R and Yoshino T: Potent synergy of dual antitumor peptides for growth suppression of human glioblastoma cell lines. Mol Cancer Ther 7: 1461-1471, 2008.
23. Morris MC, Depollier J, Mery J, Heitz F and Divita G: A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19: 1173-1176, 2001.
24. Fahraeus R, Lain S, Ball KL and Lane DP: Characterization of the cyclin-dependent kinase inhibitory domain of the INK4 family as a model for a synthetic tumour suppressor molecule. Oncogene 16: 587-596, 1998.
25. Arvanitis DA and Spandidos DA: Deregulation of the G1/S phase transition in cancer and squamous intraepithelial lesions of the uterine cervix: a case control study. Oncol Rep 20: 751-760, 2008.
26. Kwon TK, Buchholz MA, Chrest FJ and Nordin AA: Staurosporine-induced G1 arrest is associated with the induction and accumulation of cyclin-dependent kinase inhibitors. cell Growth Differ 7: 1305-1313, 1996.
27. Donnellan R and Chetty R: Cyclin D1 and human neoplasia. Mol Pathol 51: 1-7, 1998.
28. Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501-1512, 1999.
29. Ikuerowo SO, Kuczyk MA, von Wasielewski R, Shittu OB, Janas U, Machtens S, Serth J: p16INK4a expression and clinicopathologic parameters in renal cell carcinoma. Eur Urol 51: 732-737, 2007.
30. Scharml P, Struckmann K, Bendnar R, Fu W, Gasser T, Wilber K, Kononen J, Sauter G, Mihatsch MJ and Moch H: CDKNA2A mutation analysis, protein expression, and deletion mapping of chromosome 9p in conventional clear-cell renal carcinomas: evidence for a second tumor suppressor gene proximal to CDKN2A. Am J Pathol 158: 593-601, 2001.
31. Kawada Y, Nakamura M, Ishida E, Shimada K, Oosterwijk E, Uemura H, Hirao Y, Chul KS and Konishi N: Aberrations of the p14(ARF) and p16(INK4a) genes in renal cell carcinomas. Jpn J Cancer Res 92: 1293-1299, 2001.

BJUI
BJU INTERNATIONAL

Maintenance therapy with bacillus Calmette-Guérin Connaught strain clearly prolongs recurrence-free survival following transurethral resection of bladder tumour for non-muscle-invasive bladder cancer

Shiro Hinotsu[1], Hideyuki Akaza[2], Seiji Naito[3], Seiichiro Ozono[4], Yoshiteru Sumiyoshi[5], Sumio Noguchi[6], Akito Yamaguchi[7], Satoshi Nagamori[8], Akito Terai[9], Yasutomo Nasu[10], Haruki Kume[11], Yoshihiko Tomita[12], Yoshinori Tanaka[13], Shoji Samma[14], Hirotsugu Uemura[15], Hirofumi Koga[7] and Tomoyasu Tsushima[16]

[1]Kyoto University, Kyoto, [2]Research Centre for Advanced Science and Technology The University of Tokyo, Tokyo, [3]Kyushu University Hospital, Fukuoka, [4]Hamamatsu University School of Medicine, University Hospital, Hamamatsu, [5]National Hospital Organization Shikoku Cancer Centre, Matsuyama, [6]Yokosuka Kyousai Hospital, Yokosuka, [7]Harasanshin Hospital, Fukuoka, [8]National Hospital Organization Hokkaido Cancer Centre, Sapporo, [9]Kurashiki Central Hospital, Kurashiki, [10]Okayama University Hospital, Okayama, [11]The University of Tokyo Hospital, Tokyo, [12]Yamagata University Hospital, Yamagata, [13]Japan Red Cross Musashino Hospital, Musashino, [14]Nara Prefectural Nara Hospital, Nara, [15]Kinki University Hospital, Osaka, and [16]Okayama Medical Centre, Okayama, Japan

Accepted for publication 21 July 2010

Study Type – Therapy (RCT)
Level of Evidence 1b

OBJECTIVE

- To confirm the recurrence-preventing efficacy and safety of 18-month bacillus Calmette-Guérin (BCG) maintenance therapy for non-muscle-invasive bladder cancer.

PATIENTS AND METHODS

- The enrolled patients had been diagnosed with recurrent or multiple non-muscle-invasive bladder cancer (stage Ta or T1) after complete transurethral resection of bladder tumours (TURBT).
- The patients were randomized into three treatment groups: a maintenance group (BCG, 81 mg, intravesically instilled once weekly for 6 weeks as induction therapy, followed by three once-weekly instillations at 3, 6, 12 and 18 months after initiation of the induction therapy), a non-maintenance group (BCG, 81 mg, intravesically instilled once weekly for 6 weeks) and an epirubicin group (epirubicin, 40 mg, intravesically instilled nine times). The primary endpoint was recurrence-free survival (RFS).

RESULTS

- Efficacy analysis was performed for 115 of the full-analysis-set population of 116 eligible patients, including 41 maintenance group patients, 42 non-maintenance group patients and 32 epirubicin group patients.
- At the 2-year median point of the overall actual follow-up period, the final cumulative RFS rates in the maintenance, non-maintenance and epirubicin groups were 84.6%, 65.4% and 27.7%, respectively.
- The RFS following TURBT was significantly prolonged in the maintenance group compared with the non-maintenance group (generalized Wilcoxon test, $P = 0.0190$).

CONCLUSION

- BCG maintenance therapy significantly prolonged the post-TURBT RFS compared with BCG induction therapy alone or epirubicin intravesical therapy.

KEYWORDS

bacillus Calmette-Guérin, maintenance therapy, non-muscle-invasive bladder cancer, intravesical therapy, transurethral resection, prevention of recurrence

INTRODUCTION

BCG intravesical instillation is the most effective immunotherapy for intermediate and high-risk non-muscle-invasive bladder cancer (NMIBC) [1–7]. Lamm *et al.* [1] reported in the Southwest Oncology Group (SWOG) 8507 study that 3-year BCG maintenance therapy markedly prolonged the recurrence-free survival (RFS) and time to disease progression in comparison with the conventional 6-week induction therapy. Since that report, other randomized, comparative clinical studies have been carried out, but they used different treatment regimens and were unable to verify the efficacy of BCG maintenance therapy [8–10]. To date, the SWOG 8507 study has been the only report to

clearly demonstrate the efficacy of BCG maintenance therapy by means of a randomized comparative clinical trial. In consideration of the present situation, Herr [11] recently argued that we have not yet generated sufficient evidence to support the efficacy of BCG maintenance therapy for bladder cancer.

In the SWOG 8507 study, stratified analysis of the efficacy of BCG maintenance therapy in preventing disease recurrence in the study, which enrolled patients with recurrent or multiple stage Ta or T1 NMIBC and patients with carcinoma *in situ*, showed that the efficacy was most striking in patients with stage Ta and T1 disease [1]. However, that study was not controlled by inclusion of either a transurethral resection of bladder tumour (TURBT) monotherapy group or an anti-cancer drug instillation group. In addition, it was reported that the maintenance therapy had to be discontinued in many patients because of exacerbation of drug-related adverse events (AEs) and that only 16% of the patients were able to complete the full treatment schedule covering 36 months [1]. With regard to clinical research performed in Japan, the Japanese Urological Cancer Research Group carried out a combined analysis and reported that recurrence manifested within 500 days of surgery in most patients with stage Ta or T1 bladder cancer [12].

The present clinical trial was designed to compare and confirm the recurrence-preventing efficacy of BCG Connaught strain maintenance therapy in patients with stage Ta or T1 bladder cancer. A randomized, comparative clinical study was designed that included three treatment groups: a BCG maintenance group, a non-maintenance group and an epirubicin (EPI) group as an active control. The duration of the BCG maintenance therapy was set at 18 months because that period was thought to present the greatest risk of disease recurrence [12] and would also make it possible to achieve a certain level of treatment compliance.

PATIENTS AND METHODS

The protocol for this clinical trial was approved by the Institutional Review Board of each participating institution. In addition, the trial was initiated only after having obtained written informed consent from each of the enrolled patients, and it was conducted in accordance with good clinical practice [13].

*FIG. 1. Study design. *Patients who received three or fewer intravesical instillations during the induction therapy were not to be advanced to the maintenance therapy. **Even in the case that each instillation cycle in the induction or maintenance therapy could not be completed because of local bladder symptoms or systemic symptoms following an instillation, it was considered acceptable to carry out the maintenance therapy provided that the symptoms resolved before starting the next cycle of maintenance therapy.*

Patients who had been diagnosed with recurrent or multiple tumours of bladder cancer, had undergone excision of all of their tumours by TURBT, and were confirmed to have stage Ta or T1 transitional cell carcinoma of the bladder by histopathological analyses were included in this trial if they fell into any of the following three categories: (a) the presence of at least three bladder tumours at the time of TURBT (b) the present recurrence is at least the third such event or (c) the present recurrence was diagnosed within 12 months from the date of previous TURBT for the NMIBC.

The patients satisfied all of the following inclusion criteria: age between 20 and 79 years; no history of intravesical instillation of BCG or an anthracycline anti-tumour drug within the 12-month period following the day on which TURBT was performed; one course of BCG treatment that had been performed more than 12 months earlier was permitted; for patients who had been intravesically administered an anti-cancer drug such as mitomycin C or cytarabine or had received an oral antimetabolite, a washout period of at least 4 weeks was required, but administration of other anti-cancer treatments after the present TURBT was not permitted; white blood cell count 3300/mm^3 to 10 000/mm^3; platelet count $\geq 10 \times 10^4$/mm^3; haemoglobin: ≥9.5 g/dL; aspartate and alanine aminotransferases no more than double the upper limit of normal for the performing institution; serum total bilirubin ≤2 mg/dL; serum creatinine ≤1.5 mg/dL; and Eastern Cooperative Oncology Group performance status 0–2.

In addition, patients were excluded from this trial if they met any of the following exclusion criteria: presence of carcinoma *in situ* lesion in the bladder; stage T2 or higher muscle-invasive bladder cancer, or a history thereof; presence of a malignant tumour of the upper urinary tract or the urethra as a complication, or a history thereof; a history of intravenous administration of an anti-cancer agent or intra-arterial chemotherapy for bladder cancer, or a history of radiation therapy; currently being administered such drug therapies as an immunosuppressive dose of a steroid; congenital or acquired immunodeficiency; active double cancers; showing a strongly positive tuberculin reaction; active tuberculosis or currently undergoing treatment with anti-tubercular drugs; serious bacterial infections of the urinary tract or, in the 4 weeks before registration for this trial, a serious viral or bacterial infection; a history of serious hypersensitivity or having some other serious complication; presence of a contracted bladder; female patients who are lactating or may be pregnant; or other patients whom the investigator considers to be inappropriate for inclusion in the study.

Figure 1 shows the study design. Patients who had undergone excision of all of their bladder

tumours by TURBT were registered with the Central Registration Centre. The centre then randomly allocated them to three treatment groups: a BCG maintenance group (hereinafter, 'maintenance group'), a BCG induction monotherapy group (hereinafter, 'non-maintenance group') and an EPI group.

The allocation was performed by a minimization method [14] that employed the main background factors (primary and multiple tumours, recurrent and solitary tumours, and recurrent and multiple tumours; whether or not BCG intravesical instillation had been performed previously; whether or not anthracycline anti-tumour drug intravesical instillation had been performed previously; the institution) as allocation factors.

Treatment of each patient was to be started no later than within 1 month following TURBT. For the patients in the non-maintenance group, 81 mg of the BCG Connaught strain (ImmuCyst®; manufactured by Sanofi Pasteur Ltd., Toronto, Canada) was first suspended in 3 mL diluent, followed by dilution with 40 mL physiological saline. That suspension was then instilled into the bladder via a urethral catheter, and the patient was instructed to endeavour to retain it for 2 h, and only then to urinate. This intravesical instillation procedure was to be performed once weekly, for a total of six treatments. For the maintenance group, the 6-week induction therapy (i.e. the above BCG instillation protocol) was performed first, and then maintenance therapy was carried out by performing additional BCG intravesical instillations according to a schedule of once weekly for 3 weeks, at each of 3, 6, 12 and 18 months from the date when the induction therapy had been started. EPI (Farmorubicin®; Pfizer Japan, Tokyo, Japan), 40 mg, was dissolved in 40 mL physiological saline and instilled into the bladder. The patient was to retain that solution for 1–2 h and then urinate. This EPI intravesical instillation was performed twice at a 1-week interval and then seven times at 2-week intervals. This administration regimen was adopted from that reported by the Japanese Urological Cancer Research Group for a series of clinical studies [15–17] and which had yielded good results [17].

Efficacy of the treatment regimens was assessed on the basis of cystoscopy and urinary cytology findings. Patient follow up was performed at 3-month intervals for the first 3 years, and then at 6-month intervals beginning from the 4th year. When tumour recurrence was suspected on the basis of the findings of cystoscopy and urinary cytology, confirmation was carried out by performing TURBT. The date of confirmation of recurrence or progression was defined as the earliest date on which the diagnosis had been made based on the findings of cystoscopy, urinary cytology or some other diagnostic imaging technique.

Progression at the time of recurrence was defined as progression in the disease stage (i.e. progression to stage T2 or higher, metastasis, or appearance of tumours in the upper urinary tract or the urethra) or as exacerbation of the tumour grade.

For assessment of the safety of each of the treatment regimens, the severity of AEs was judged in accordance with the criteria stipulated in NCINational Cancer Institute-Common Toxicity Criteria, Version-2.0 [18].

The primary endpoint was the RFS from randomization and the secondary assessment endpoints were the progression rate at the time of first recurrence and the safety. The RFS was estimated by the Kaplan-Meier method. As the primary analyses, the study protocol called for two comparisons regarding the RFS. The first-step comparison was of the combined BCG group (the maintenance group plus the non-maintenance group) with the EPI group. Only if the result for the RFS was statistically superior for the combined BCG group compared with the EPI group was the second-step comparison performed, comprising a comparison of the maintenance group with the non-maintenance group. The treatment groups were compared using the generalized Wilcoxon test. The necessary number of patients in each treatment group was calculated for each comparison on the basis of assuming a registration period of 2.5 years, a study period of 4.5 years, an 80% power of the test and a 5% type one error. The expected 2-year recurrence-free rates for each group were estimated to be 85% for the maintenance group, 60% for the non-maintenance group and 45% for the EPI group, based on the earlier reported results [1,15–17]. Accordingly, the necessary numbers of patients were estimated to be 52 patients in the combined BCG group and 26 patients in the EPI group for the first-step comparison, and 36 patients in each of the treatment groups for the second-step comparison. When consideration was given to the possibility of dropouts, the total target number of patients was set at 110, consisting of 40 patients in each of the maintenance and non-maintenance groups and 30 patients in the EPI group. For unequal treatment allocation, the biased-coin minimization method [19] was used in the allocation programme. Smoothed hazards analysis [7,12,20] was applied to estimate the hazard of recurrence in each of the treatment groups.

There had initially been no plan to perform an interim test for significant differences during the course of this trial. However, as the trial progressed, it became evident that there were more recurrent cases in the EPI group than had been expected at the start. This raised the question of whether, from an ethical standpoint, the trial should be terminated earlier than scheduled. To address this issue, the Independent Efficacy and Safety Assessment Committee for this trial recommended revising the analytical plans of the study. As a result, new conditions for termination of the trial were established, and it was decided to perform interim data analyses to determine the advisability of early termination. The date of cut-off for interim data analyses was set as the last day of August 2008, which was 4 years from the date of registration of the first patient in the trial instead of the initially planned 4.5 years. The main statistical test was the generalized Wilcoxon test, with a P value of 0.035 indicating statistical significance, which was calculated using an α consumption function that permitted adjustment for the type one error for the study as a whole.

RESULTS

Figure 2 explains the grouping of the patients in this clinical trial. A total of 120 patients were enrolled in the trial at 37 participating institutions between August 2004 and the end of December 2006. Following registration and before the start of treatment, four patients were excluded. The enrolled patients were randomly allocated to the three treatment groups after registration, and a total of 116 patients were started on their respective treatments. One of the patients in the maintenance group retracted consent and was excluded from the efficacy analysis.

Hence, the 'full analysis set' analytical population numbered 115 patients in total.

Analysis of the per protocol set analytical population was carried out as a secondary endpoint of analysis. The study protocol stipulated that, for a maintenance group patient to be included in the per protocol set analysis, it was necessary for the patient to have been administered at least one BCG intravesical instillation as maintenance therapy.

Table 1 presents the data on the background characteristics of the FAS population.

Figure 3 shows, the respective RFS plots for the combined BCG group and the EPI group, and for the maintenance group and the non-maintenance group.

In the first step analysis, the generalized Wilcoxon test showed that the RFS was significantly prolonged in the combined BCG group compared with the EPI group ($P < 0.0001$). Accordingly, comparison of the RFS between the maintenance group and the non-maintenance group was carried out as the second step analysis. That analysis showed the RFS to be significantly prolonged in the maintenance group compared with the non-maintenance group (generalized Wilcoxon test: $P = 0.0190$). This result for the main analyses satisfies the criterion for statistical significance that was stipulated for the interim analyses. For that reason, this result was considered to represent the final analysis, and the clinical trial was terminated early.

In the comparison of the per protocol set analytical population, only two of the 36 patients in the maintenance group experienced recurrence. That analysis also showed the RFS to be significantly prolonged in the maintenance group compared with the non-maintenance group (generalized Wilcoxon test: $P = 0.0007$).

Figure 4 presents the results of smoothed hazards analysis of the recurrence in each of the treatment groups. In the EPI group, a high recurrence hazard continued for 2 years after TURBT, whereas in the non-maintenance group the recurrence hazard decreased from the half-year point onward. On the other hand, in the maintenance group, in addition to suppressing the initial hazard to about half of that in the non-maintenance group, a low hazard was maintained for up to approximately 2 years after TURBT.

FIG. 2. Accounting of the patients; CONSORT diagram.

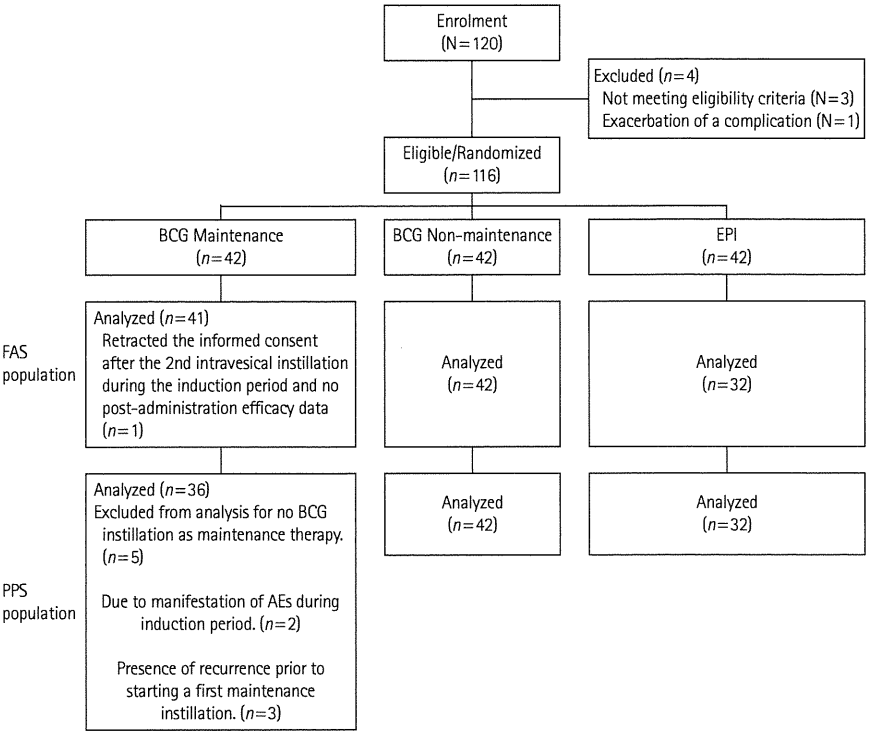


Table 2 presents the data on the numbers of patients in each of the treatment groups who showed progression at the time of first recurrence. The progression rates were compared between the combined BCG group and the EPI group and between the maintenance group and the EPI group. Fisher's exact test showed statistically significant differences in those comparisons ($P = 0.0047$ and $P = 0.0021$, respectively). When the maintenance group and the non-maintenance group were then compared, no statistically significant difference was found (Fisher's exact test: $P = 0.2410$). However, there were no cases of progression in the maintenance arm.

The incidence of treatment-related AEs was higher in the maintenance group than in the non-maintenance group (Table 3). However, all of these symptoms could be controlled by suspending the treatment or administering therapy such as an anti-inflammatory or analgesic. Even for the maintenance treatment cycle, at the 18-month point approximately 70% of the patients were able to be given at least one of the three planned instillations (Table 4).

The incidence of treatment-related AEs was clearly lower in the EPI group patients compared with in the BCG groups. Grade 3 or more severe treatment-related AEs consisted of gross haematuria (3.1%) and malaise (3.1%).

DISCUSSION

The SWOG 8507 study did not include either a TURBT-only group or a chemotherapeutic drug instillation group. Our present study included an EPI group as an active control group. The design made it possible to clearly show the difference in efficacy among the three treatment groups. The 2-year RFS rate for the BCG maintenance group was 92.7%, which was markedly better than the rates of 65.4% for the non-maintenance group and 33.2% for the EPI group. Two-year RFS rates of approximately 80% and 60% were estimated for the maintenance group and the non-maintenance group in the SWOG 8507

study, respectively [1]. In addition, Saint *et al.* [21] carried out a clinical study that administered the BCG Connaught strain according to a 3-year maintenance schedule, and they reported a 2-year RFS rate of 84.9%. Furthermore, van der Meijden *et al.* [22] reported an estimated 2-year RFS rate of 70% with maintenance therapy that employed the BCG Tice strain. It is clear that the 92.7% 2-year RFS rate was reported in our study is significant and similar to the results in those earlier studies.

TABLE 1 Patient background characteristics (full analysis set population)

Characteristic	BCG* 83	EPI 32	*P* value	M 41	Non-M 42	*P* value†
Stage						
pTa	58	24	0.6515	29	29	1.0000
pT1	25	8		12	13	
Age						
≤64	39	11	0.2944	17	22	0.3817
≥65	44	21		24	20	
Sex						
Male	73	31	0.2861	33	40	0.0485
Female	10	1		8	2	
Previous Anthracycline‡						
No	69	26	0.7895	33	36	0.5700
Yes	14	6		8	6	
Previous BCG‡						
No	77	29	0.7072	38	39	1.0000
Yes	6	3		3	3	
Grade						
G1	15	4	0.7541	5	10	0.3966
G2	53	21		29	24	
G3	15	7		7	8	
History/Multiplicity‡						
Recurrent/Multiple	41	15	0.7023	20	21	1.0000
Primary/Multiple	36	13		18	18	
Recurrent/Solitary	6	4		3	3	
EORTC Recurrence score						
Intermediate risk (1–9)	72	25	0.2637	36	36	1.0000
High risk (≥10)	11	7		5	6	

*M, maintenance group; Non-M, non-maintenance group; EORTC, European Organization for the Research and Treatment of Cancer. *Combination of the maintenance group and non-maintenance group; †Fisher's exact test; ‡Allocation factors: primary and multiple tumours, recurrent and solitary tumours, and recurrent and multiple tumours; previous history of BCG intravesical instillation; previous history of anthracycline intravesical; the institution.*

The SWOG 8507 study found that maintenance therapy significantly prolonged the period until disease progression (log rank test, $P = 0.04$) [1]. In addition, Bohle and Bock [23] performed a meta-analysis of 2410 patients to compare the efficacy of BCG and mitomycin C in suppressing disease progression. They reported that administration of BCG maintenance therapy for at least 1 year resulted in significantly superior suppression of the risk of disease progression that was 34% greater than that with mitomycin C (OR = 0.66; 95% CI 0.47–0.94; $P = 0.02$). Moreover, Sylvester *et al.* [24] carried out a meta-analysis of 4863 patients in which they compared the therapeutic results in a BCG group, a TURBT-only group and an anti-cancer drug group. They reported that the BCG group showed statistically significant suppression of disease progression (OR = 0.73; 95% CI 0.60–0.89; $P = 0.001$). Also, when the analysis was performed for patients who received some form of BCG maintenance therapy for at least 1 year, striking efficacy was demonstrated (OR = 0.63; 95% CI 0.51 $P = 0.79$; $P = 0.00004$). In addition, a recent systematic review [25] by the Cochrane group concluded that BCG maintenance significantly reduced recurrence in patients with non-muscle-invasive bladder cancer. On the basis of these and other reports, the guidelines for treatment of bladder cancer all recommend that BCG maintenance therapy be continued for at least 1 year [26–31].

On the other hand, Herr [11] recently argued that there is insufficient evidence indicating that BCG maintenance therapy shows

TABLE 2 Numbers of patients showing progression at time of first recurrence (full analysis set)

	No. of patients	Patients with recurrence, *n* (%)	Patients with progression, *n* (%)	Nature of progression		
				Stage progression to ≥T2	Extracystic progression*	Exacerbation of tumour grade
Maintenance group	41	5 (12.2)	0 (0)	0	0	0
Non-maintenance group	42	14 (33.3)	3 (7.1)	0	2†	2†
Epirubicin group	32	22 (68.8)	7 (21.9)	1	2	4

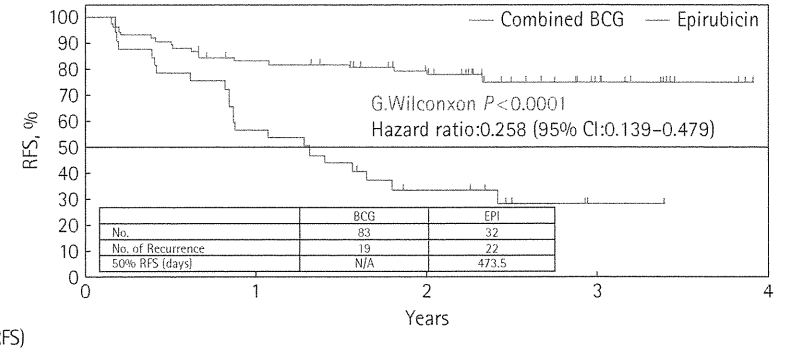
**Metastasis or tumour expression into upper urinary tract or urethra; †one of these patients is the same.*

significant efficacy in suppressing disease progression. However, most events of progression are detected during the course of repeated recurrence in the bladder, and it can therefore be thought that achieving RFS over a long period of time by performing maintenance therapy will also reduce the risk of progression. Actually, in our study no progression was observed in the maintenance group. The efficacy results for BCG maintenance therapy that we have demonstrated in our present randomized trial not only support the findings reported by Lamm *et al.* [1] but are also able to partially counter the recent discussion regarding BCG maintenance therapy [11,32–34].

For the tolerability evaluation of 81 mg of BCG dose, our present protocol stipulated that the dose of BCG could not be reduced, because one objective of the study was to confirm the tolerability of BCG when administered in a dose of 81 mg. Treatment-related AEs were clearly more severe during the maintenance therapy. It was reported that treatment-related AEs could be reduced, without sacrificing efficacy, by lowering the BCG dose [35,36] or by administering a quinolone antibiotic 8 h after BCG instillation [21]. We think that future studies are warranted to investigate the possibility of further increasing the duration of long-term prevention of bladder cancer recurrence by improving treatment compliance through better management of treatment-related AEs during the course of BCG maintenance therapy.

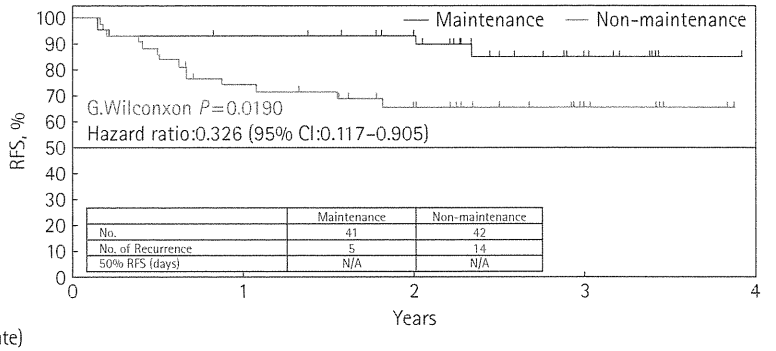
In conclusion, this randomized, comparative clinical trial demonstrated that BCG intravesical instillation maintenance therapy is able to significantly prolong post-TURBT RFS in patients with recurrent or multiple, stage Ta or T1, bladder cancer that is at moderate-to-high risk of recurrence. The results of this clinical trial represent valuable new evidence in support of the efficacy of BCG maintenance therapy in preventing bladder cancer recurrence.

FIG. 3. Recurrence-free survival. Plot of Kaplan-Meier estimates for recurrence-free survival (FAS population) for the combined BCG group and EPI group (upper), and for the maintenance group and non-maintenance group (lower).



at risk (RFS)

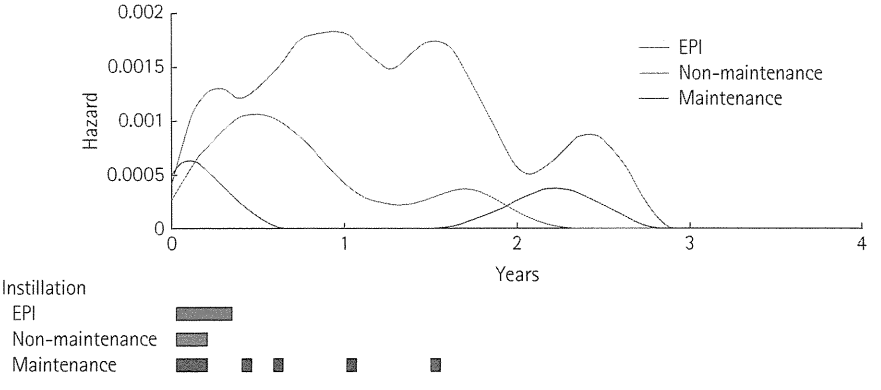
Combined BCG	83	66(83.1)	51(79.0)	16(75.3)
Epirubicin	32	18(56.3)	8(33.2)	1(27.7)



at risk (RFS rate)

Maintenace	41	37(92.7)	30(92.7)	9(84.6)
Non-maintenance	42	29(73.6)	21(65.4)	7(65.4)

FIG. 4. Smoothed hazards analysis of recurrence.



ACKNOWLEDGEMENTS

We appreciate the cooperation afforded by all of the patients and institutions involved in this study. In Japan, neither the usage of BCG adjuvant treatment after TURBT nor a schedule of maintenance therapy for NMIBC has yet been approved. Accordingly, the present clinical study was carried out with the objective of supporting an approval application for BCG maintenance therapy for preventing the recurrence of NMIBC in Japan. The trial is supported by Nippon Kayaku Co. Ltd (Tokyo, Japan), which is the current Japanese licence holder for the BCG Connaught strain.

CONFLICT OF INTEREST

None declared.

TABLE 3 The major treatment-related adverse events (AEs) in the maintenance group and the non-maintenance group

	Maintenance arm ($N = 42$)*			Non-maintenance arm ($N = 42$)*		
Event	Pts *n*	Incidence (%)	≥Grade 3 (%)	Pts *n*	Incidence (%)	≥Grade 3 (%)
Urinary frequency	39	92.9	40.5	30	71.4	19.0
Pain on urination	39	92.9	9.5	29	69.0	2.4
Difficulty in urination	21	50.0	4.8	12	28.6	
Gross haematuria	39	92.9	19.0	30	71.4	11.9
Residual urine	8	19.0		2	4.8	
Bladder pain	3	7.1	2.4	0		
Lower abdominal pain	4	9.5		2	4.8	2.4
Epididymitis	1	2.4	2.4	0		
Bladder tamponade	1	2.4	2.4	0		
Pyrexia (≥38 °C)	18	42.9		11	26.2	
Malaise	21	50.0		18	42.9	
Anorexia	13	31.0		4	9.5	
Arthralgia	7	16.7		4	9.5	4.8
Headache	5	11.9		3	7.1	
Hypertension	1	2.4	2.4	0		
Leukocytosis†	6	14.3		3	7.1	
Urinary protein positive	20	47.6		10	23.8	
Microscopic haematuria†	31	73.8		25	59.5	
Urinary red blood cell increase†	29	69.0		24	57.1	
Urinary white blood cell increase†	36	85.7		31	73.8	

*The severity of AEs was judged in accordance with the criteria stipulated in the Japanese version of the Japan Clinical Oncology Group 2nd edition of the National Cancer Institute – Common Toxicity Criteria version 2.0. *N represents the number of patients for whom safety was evaluable; †not graded. Pts, Patients.*

TABLE 4 Completion rates and performance rates for the maintenance therapy cycles in the patients advanced to the maintenance therapy

		Performance rate		Completed rate	
Cycle	*N*	*n*	%	*n*	%
At 3 months	36	36	100.0	32	88.9
At 6 months	36	35	97.2	23	63.9
At 12 months	36	30	83.3	19	52.8
At 18 months	36	25	69.4	15	41.7

The completion rate (i.e. all three of the planned instillations in the cycle were administered) and performance rate (i.e. at least one of the three planned instillations in the cycle was administered) are shown for each of the instillation cycles at the 3-, 6-, 12- and 18-month points of the maintenance therapy for the 36 patients who were advanced to that therapy.

REFERENCES

1 **Lamm DL, Blumenstein BA, Crissman JD *et al.*** Maintenance bacillus Calmette-Guérin immunotherapy for recurrent TA, T1 and carcinoma *in situ* transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. *J Urol* 2000; **163**: 1124–9

2 **Lamm DL, van der Meijden APM, Akaza H *et al.*** Intravesical chemotherapy and immunotherapy: how do we assess their effectiveness and what are their limitations and uses? *Int J Urol* 1995; **2** (Suppl. 2): 23–35

3 **Kurth KH, Bouffioux C, Sylvester R *et al.*** Treatment of superficial bladder tumors: achievement and needs. *Eur Urol* 2000; **37** (Suppl. 3): 1–9

4 **Akaza H, Koiso K, Ozono S *et al.*** A clinical study of PMCJ-9 (Bacillus Calmette-Guérin Connaught strain) treatment of superficial bladder cancer and carcinoma *in situ* of the bladder. *Jpn J Clin Oncol* 2003; **33**: 382–90

5 **Bohle A, Jocham D, Bock PR.** Intravesical bacillus Calmette-Guérin versus Mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity. *J Urol* 2003; **169**: 90–5

6 **Han RF, Pan JG.** Can intravesical bacillus Calmette-Guérin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials. *Urology* 2006; **67**: 1216–23

7 **Hinotsu S, Akaza H, Isaka S *et al.*** Sustained prophylactic effect of intravesical bacille Calmette-Guérin for superficial bladder cancer: a smoothed hazard analysis in a randomized prospective study. *Urology* 2006; **67**: 545–9

8 **Badalament RA, Herr HW, Wong GY *et al.*** A prospective randomized trial of maintenance versus nonmaintenance intravesical bacillus Calmette-Guérin therapy of superficial bladder cancer. *J Clin Oncol* 1987; **5**: 441–9

9 **Palou J, Laguna P, Millán-Rodríguez F,**

Hall RR, Salvador-Bayarri J, Vicente-Rodríguez J. Control group and maintenance treatment with bacillus Calmette-Guérin for carcinoma *in situ* and/or high grade bladder tumors. *J Urol* 2001; **165**: 1488–91

10 **Hudson MA, Ratliff TL, Gillen DP, Haaff EO, Dresner SM, Catalona WJ.** Single course versus maintenance bacillus Calmette-Guérin therapy for superficial bladder tumors: a prospective, randomized trial. *J Urol* 1987; **138**: 295–8

11 **Herr HW.** Is maintenance Bacillus Calmette-Guérin really necessary? *Eur Urol* 2008; **54**: 971–3

12 **Hinotsu S, Akaza H, Ohashi Y, Kotake T.** Intravesical chemotherapy for maximum prophylaxis of new early phase superficial bladder carcinoma treated by transurethral resection: a combined analysis of trials by the Japanese Urological Cancer Research Group using smoothed hazard function. *Cancer* 1999; **86**: 1818–26

13 **The Japan Pharmaceutical Manufacturers Association.** *Japanese Ministerial Ordinance on Standards for Conducting Clinical Trials on Pharmaceuticals.* Tokyo: Elsevier Japan K.K. 1997 Ordn. (MHW), No. 28 (in Japanese)

14 **Pocock SJ, Simon R.** Sequential treatment assignment with balancing for prognostic factors in the controlled clinical trials. *Biometrics* 1975; **31**: 103–15

15 **Akaza H, Isaka S, Koiso K *et al.*** Comparative analysis of short-term and long-term prophylactic intravesical chemotherapy of superficial bladder cancer. Prospective, randomized, controlled studies of the Japanese Urological Cancer Research Group. *Cancer Chemother Pharmacol* 1987; **20** (Suppl.): S91–6

16 **Hinotsu S, Akaza H, Isaka S *et al.*** Japanese Urological Cancer Research Group for Adriamycin/Farmorubicin. Intravesical instillation of doxorubicin or epirubicin for chemoprophylaxis of superficial bladder cancer – the fifth study of the Japanese Urological Cancer Research Group for Adriamycin/Farumorubicin]. *Jpn J Cancer Chemother* 2002; **29**: 73–80 (in Japanese)

17 **Kuroda M, Niijima T, Kotake T, Akaza H, Hinotsu S.** Effect of prophylactic treatment with intravesical epirubicin on recurrence of superficial bladder cancer: the 6th trial of the Japanese Urological Cancer Research Group (JUCRG) – a randomized trial of intravesical epirubicin at dose of 20 mg/40 ml, 30 mg/40 ml, 40 mg/40 ml. *Eur Urol* 2004; **45**: 600–5

18 **Japan Clinical Oncology Group.** *National Cancer Institute-Common Toxicity Criteria (NCI-CTC)Version 2.0. Japanese Version of Japan Clinical Oncology Group 2nd Edn.* 2001

19 **Han B, Enas NH, McEntegart D.** Randomization by minimization for unbalanced treatment allocation. *Stat Med* 2009; **28**: 3329–46

20 **Gray RJ.** Some diagnostic methods for Cox regression models through hazard smoothing. *Biometrics* 1990; **46**: 93–102

21 **Saint F, Irani J, Patard JJ *et al.*** Tolerability of bacille Calmette-Guérin maintenance therapy for superficial bladder cancer. *Urology* 2001; **57**: 883–8

22 **van der Meijden AP, Brausi M, Zambon V, Kirkels W, de Balincourt C, Sylvester R.** Members of the EORTC Genito-Urinary Group. Intravesical instillation of epirubicin, bacillus Calmette-Guérin and bacillus Calmette-Guérin plus isoniazid for intermediate and high risk Ta, T1 papillary carcinoma of the bladder: a European Organization for Research and Treatment of Cancer genito-urinary group randomized phase III trial. *J Urol* 2001; **166**: 476–81

23 **Bohle A, Bock PR.** Intravesical bacillus Calmette-Guérin versus Mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumor progression. *Urology* 2004; **63**: 682–7

24 **Sylvester RJ, van der Meijden APM, Lamm DL.** Intravesical Bacillus Calmette-Guérin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials. *J Urol* 2002; **168**: 1964–70

25 **Shelley MD, Mason MD, Kynaston H.** Intravesical therapy for superficial bladder cancer: a systematic review of randomized trials and meta-analyses. *Cancer Treat Rev* 2010; **36**: 195–205

26 **Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Bohle A, Palou J.** Guidelines on TaT1 (Non-muscle invasive) Bladder Cancer. European Association of Urology (EAU). *Eur Urol* 2008; **54**: 303–14

27 **American Urological Association.** Guideline for the management of nonmuscle invasive bladder cancer: (Stage Ta, T1, and Tis): 2007 Update. [homepage on the internet]. Available at:http://www.auanet.org/content/guidelines-and-quality-care/clinical-guidelines.cfm?sub=bc. Accessed 11 August 2010

28 **Sylvester RJ, van der Meijden A, Witjes JA *et al.*** High-grade Ta urothelial carcinoma and carcinoma in *situ of* the bladder. *Urology* 2005; **66**: 90–107

29 **National Comprehensive Cancer Network.** NCCN Clinical Practice Guideline in Oncology TM: Bladder Cancer: Including upper tract tumors and urothelial carcinoma of the prostate v.I.2009. [homepage on the internet]. Available at:http://www.nccn.org/professionals/physician_gls/PDF/bladder.pdf. Accessed 31 January 2009

30 **The Japanese Urological Association.** *Bladder Cancer Clinical Practice Guideline.* Tokyo: Igaku Tosho Syuppan Co., Ltd., Tokyo, 2009: (in Japanese)

31 **Lamm DL, Colombel M, Persad R *et al.*** Clinical practice recommendations for the management of non-muscle invasive bladder cancer. *Eur Urol* 2008; (Suppl. 7): 651–66

32 **Malmström PU, Sylvester RJ, Crawford DE *et al.*** An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus bacillus Calmette-Guérin for non-muscle-invasive bladder cancer. *Eur Urol* 2009; **56**: 247–56

33 **Lamm D, Böhle A, Palou J *et al.*** Re: Per-Uno Malmström, Richard J. Sylvester, David E. Crawford *et al.* An individual patient data meta-analysis of the long-term outcome of randomised studies comparing intravesical mitomycin C versus bacillus Calmette-Guérin for non-muscle-invasive bladder cancer. *Eur Urol* 2009; **56**: 247–56. *Eur Urol 2010*; 57: e7–9; author reply e10–1

34 **Gontero P, Bohle A, Malmstrom PU *et al.*** The role of bacillus Calmette-Guérin in the treatment of non-muscle-invasive bladder cancer. *Eur Urol* 2010; **57**: 410–29

35 **Martínez-Piñeiro JA, Martínez-Piñeiro L, Solsona E *et al.*** Club Urológico Español de Tratamiento Oncológico (CUETO). Has a 3-fold decreased dose of bacillus Calmette-Guérin the same efficacy against recurrences and progression of T1G3 and Tis bladder tumors than the standard dose? Results of a prospective randomized trial. *J Urol* 2005; **174**: 1242–7

36 **Mugiya S, Ozono S, Nagata M *et al.*** Long-term outcome of a low-dose

intravesical bacillus Calmette-Guérin therapy for carcinoma *in situ* of the bladder: results after six successive instillations of 40 mg BCG. *Jpn J Clin Oncol* 2005; **35**: 395–9

Correspondence: Hideyuki Akaza, Department of Strategic Investigation on Comprehensive Cancer Network, Research Centre for Advanced Science and Technology, The University of Tokyo 4-6-1, Komaba, Meguro-ku, Tokyo 153-8904, Japan.
e-mail: akazah@med.rcast.u-tokyo.ac.jp

Abbreviations: **NMIBC**, non-muscle-invasive bladder cancer; **RFS**, recurrence-free survival; **SWOG**, Southwest Oncology Group; **TURBT**, transurethral resection of bladder tumour; **AE**, adverse event; **EPI**, epirubicin.

APPENDIX

The authors wish to thank the investigators for performing this study. Investigators and institutions: T. Tsukamoto, Sapporo Medical University Hospital; T. Ohyama, Hirosaki University School of Medicine and Hospital; A. Ito, Tohoku University Hospital; N. Tsuchiya, Akita University Hospital; N. Miyanaga, Tsukuba University Hospital; K. Tanabe, Tokyo Women's Medical University Hospital; E. Kikuchi, Keio University Hospital; I. Fukui, The Cancer Institute Hospital of JFCR; K. Tomita, The University of Tokyo Hospital; S. Egawa, The Jikei University Hospital; S. Komatsubara, Niigata Cancer Centre Hospital; S. Mugiya, Hamamatsu University School of Medicine, University Hospital; Y. Hirano, Fujieda Municipal General Hospital; H. Kobayashi, Nagoya Daini Red Cross Hospital; K. Kohri, Nagoya City University Hospital; K. Miki, University Hospital, Kyoto Prefectural University of Medicine; H. Nishiyama, Kyoto University Hospital; M. Usami, Osaka Medical Centre for Cancer and Cardiovascular Disease; N. Nonomura, Osaka University Hospital; K. Fujimoto, Nara Medical University Hospital; T. Inagaki, Wakayama Medical University Hospital; M. Takahashi, Tokushima University Hospital; M. Inui, Faculty of Medicine, Kagawa University; A. Yokomizo, Kyushu University Hospital; M. Nakagawa, Kagoshima University Medical and Dental Hospital. The Protocol and Evaluation Committee: Y. Kubota, Yokohama City University Hospital; T. Habuchi, Akita University Hospital; H. Kanetake, Nagasaki University Hospital. Coordinating Committee: T. Tsukamoto, Sapporo Medical University Hospital; Y. Hirao, Nara Medical University Hospital.

The Therapeutic Effects of R8-Liposome-BCG-CWS on BBN-Induced Rat Urinary Bladder Carcinoma

JUN MIYAZAKI[1], HIROYUKI NISHIYAMA[1], IKUYA YANO[2], AKIHIRO NAKAYA[2], HIDEYASU KOHAMA[2], KOJI KAWAI[1], AKIRA JORAKU[1], TAKASHI NAKAMURA[3], HIDEYOSHI HARASHIMA[3] and HIDEYUKI AKAZA[4]

[1]*Graduate School of Comprehensive Human Sciences, Department of Urology, University of Tsukuba, Tsukuba, Ibaraki, Japan;*
[2]*Japan BCG Central Laboratory, Kiyose, Tokyo, Japan;*
[3]*Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido, Japan;*
[4]*Department of Strategic Investigation on Comprehensive Cancer Network, Research Center for Advanced Science and Technology, University of Tokyo, Japan*

Abstract. *Background: The present gold standard for bladder cancer is Mycobacterium bovis bacillus Calmette-Guérin (BCG) immunotherapy, but serious side-effects are common. We previously reported that C3H/HeN mice vaccinated with a mixture of MBT-2 cells and artificial BCG, octaarginine-modified liposomes incorporating the cell wall of BCG (R8-liposome-BCG-CW), significantly inhibited growth of R8-liposome-BCG-CW pretreated MBT-2 cells. Our aim was to determine if a non-live bacterial agent could be as efficacious as live BCG in a model of bladder cancer. We investigated the suppressive effect of liposome-incorporating cell wall skeleton (BCG-CWS) on N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced urinary bladder carcinogenesis in rats. Materials and Methods: F344 rats were fed with BBN and sodium ascorbate for 8 weeks, after which all rats were confirmed to have excreted atypical epithelial cells in the urine. Rats were administered BCG-CW(1.0 mg/rat) or R8-liposome-BCG-CWS (0.1 or 1.0 mg/rat) intravesically once/week for 8 weeks from week 28 to 35 of the experimental protocol. Results: Rats receiving R8-liposome-BCG-CWS intravesically showed significantly inhibited numbers of tumors, especially those of simple hyperplasia, in comparison with the control rats. Conclusion: R8-liposome-BCG-CWS administration had inhibitory effects on rat bladder carcinogenesis. These results may indicate a novel adoptive immunotherapy against bladder cancers.*

Correspondence to: Jun Miyazaki, Graduate School of Comprehensive Human Sciences, Department of Urology, University of Tsukuba, Tsukuba, Ibaraki, 3058575 Japan. Tel: +81 298533223, Fax: +81 298538854, e-mail: jmiyazaki@md.tsukuba.ac.jp

Key Words: Bladder cancer, BCG-CWS, liposome, BBN, rat.

Intravesical BCG therapy is effective against carcinoma *in situ* and as a prophylaxis against the recurrence of bladder cancer (1-5). In addition to its direct antitumor effect, it is widely recognized that intravesical BCG therapy is more potent in preventing tumor recurrence than is intravesical chemotherapy (6). Although immunotherapy using live BCG is effective, it is not free from fatal side-effects, *e.g.*, high fever, granulomatous prostatitis, pneumonitis, hepatitis, and BCG sepsis (7). To avoid such unfavorable events, it is necessary to develop a more active but less toxic immunotherapeutic agent.

Although the cell wall skeleton of BCG (BCG-CWS) has long been investigated for this purpose, its clinical use is very limited because of solubility and stability difficulties. To overcome these unfavorable physicochemical properties of the BCG-CWS preparation, we have applied octaarginine-modified liposomes (R8-liposomes) as a vector to transport BCG-CWS into the cytoplasm effectively. R8-liposomes were initially developed to transfer highly negatively charged DNA molecules into the cytoplasm by macropinocytosis (8-10). R8-liposomes resemble an envelope-type virus and their surface is modified by anchored R8, a characteristic and efficient cell-penetrating peptide (9).

We have previously reported that R8-liposomes-incorporating mycobacterial cell walls (R8-liposome-BCG-CW) successfully attached to the surface of MBT-2 cells and were efficiently internalized into the cytoplasm within an hour of co-incubation (11). Internalized BCG-CW was then distributed to the lysosomes of the MBT-2 cells and finally has been shown to completely inhibit the growth of MBT-2 tumors *in vivo* (11). Since the structure of BCG-CW is not stable and BCG-CWS is easy to formulate, we changed from R8-liposome-BCG-CW to R8-liposome-BCG-CWS. Furthermore, R8-liposome-BCG-CWS rendered cancer cells more susceptible to cytolysis by lymphokine-activated killer cells (12).

0250-7005/2011 $2.00+.40

However, the antitumor effect in bladder cancer has not been tested. Thus, the primary purpose of the present study was to examine the antitumor effect of R8-liposome-BCG-CWS as a single agent in a rat model carcinogen-induced of bladder carcinogenesis.

Materials and Methods

Preparation of R8-liposome-BCG-CWS. R8-liposome-BCG-CWS was prepared by a method reported previously (13).

Bladder carcinogenesis induced by BBN in rats. *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) bladder carcinogenesis is considered to be a model for superficial bladder tumor. It has been well described that during bladder cancer development, diffuse hyperplasia at 4 weeks after BBN administration is a reversible change toward cancer, whereas nodular hyperplasia at 8 weeks is an irreversible change (14).

The study comprised 20 8-week-old male Fisher-344 rats (Charles River Japan, Yokohama, Japan). All animals used in the study were handled according to the guidelines approved by the University Council for Animal Care. BBN was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The experimental protocol is summarized in Figure 1. Because sodium ascorbate significantly increases papillary or nodular hyperplasia (PN hyperplasia) (15-16), all rats were given diets containing 5% (w/w) sodium ascorbate for 8 weeks. After treatment for 8 weeks with the sodium ascorbate and 0.05% BBN in drinking water, 20 rats were divided into four groups according to the treatment administered at 28 weeks: group 1, control (phosphate-buffered saline [PBS] only); group 2, BCG-CW only (1.0 mg/rat once weekly for 8 weeks); group 3, R8-liposome-BCG-CWS (0.1mg/rat once weekly for 8 weeks), and group 4 R8-liposome-BCG-CWS (1.0 mg/rat once weekly for 8 weeks).

Histological examination. At the end of treatment, the rats were killed under anesthesia. Before removal of the bladder from each rat, the bladder was intraluminally injected with a buffered formaldehyde solution as pre-fixation for histological analyses. A ligature was placed around the bladder neck to maintain proper distention.

For macroscopic quantitative analysis (number and volume of tumors), each bladder pre-fixed in formaldehyde was carefully opened, the lumen was inspected for grossly visible lesions, and the number of tumors per rat and the volume of each tumor were recorded to calculate the incidence of tumors per group and the mean tumor volume per rat. The volume was calculated as $V\ (mm^3)=4\times\pi\times 1\ mm\ (major\ axis)\times s^2\ mm^2\ (minor\ axis)/3$. A tumor was defined as a lesion of >0.5 mm in diameter.

For microscopic qualitative analysis (bladder histology), the bladder was immersion-fixed in 4% buffered formaldehyde and processed for paraffin sectioning. Three slices from each bladder were embedded in paraffin, sliced into 3 μm sections, and stained with hematoxylin and eosin. The lesions observed in the urinary bladder mucosa were classified as simple hyperplasia, PN hyperplasia, papilloma, or carcinoma, as described previously (17).

Blood analysis. Interferon gamma (IFN-γ) in blood was analyzed in weeks 31 and 35 of the experimental protocol. We measured the serum level of IFN-γ immediately before administration of the specific treatment in each rat group and after 8 and 24 hours. Serum levels of IFN-γ were measured with a Rat IFN-γ Colorimetric ELISA Kit (Thermo Fisher Scientific, Yokohama, Japan).

Statistical analysis. The results are presented as the mean (SEM), and groups were compared using non-parametric Dunnett method with significance indicated at a value of -$p<0.05$.

Results

All rats completed the 36-week protocol, during which the mean intake of food and fluids was not significantly different among the four groups. Macroscopic appearance of bladders in the four groups is shown Figure 2. Bladders from group 1 (control) showed large tumors, but R8-liposome-BCG-CWS caused marked inhibition in bladder tumor growth.

Significant differences were present between groups 1 and 4 in numbers of tumors per rat ($-p<0.05$) (Figure 3). The mean number of tumors per rat in group 4 (4.4±1.9) was significantly lower than that in group 1 (20.0±7.6). No significant differences were evident in tumor volume among the four groups, but the total tumor volume in group 4 was less than half that in group 1 (Figure 3).

The incidences of urinary bladder tumors are summarized in Figure 4. Histological examination showed that simple and PN hyperplasia were observed in all bladders of the BBN-treated rats. The incidence of tumors in rats treated with PBS was higher than that in groups treated with BCG-CW and R8-liposome-BCG-CWS. The number of simple hyperplasias in group 4 (6.5±1.8) was significantly lower than that of group1 (24.3±6.1). The number of PN hyperplasias in group 3 was higher than that of the other groups, but statistical differences was not found among the groups. In group 1, the number of bladder papillomas and carcinomas were 1.8±2 and 1.1±1.1, respectively. The incidence of urinary bladder carcinoma in group 4 was less than that in group 1, but the difference was not significant.

IFN-γ. IFN-γ has been shown to be important in the function and maturation of multiple immune cells. IFN-γ is essential for T-helper 1 cell (Th1) immune responses and regulates T-cell differentiation, activation, expansion, homeostasis, and survival. At 31 weeks, rats in group 2 had higher levels of IFN-γ than those in other groups, but statistical differences were not found among the groups (Figure 5). At 35 weeks, IFN-γ secretion was more increased in groups 2 and 4 than in groups 1 and 3, but no significant difference was found among all groups. It is suggested that IFN-γ may be high in the groups with a low number of lesions.

Discussion

The antitumor mechanism of BCG and the role of its CWS components have recently been unveiled. Most of the immunostimulatory activities are associated with the CWS

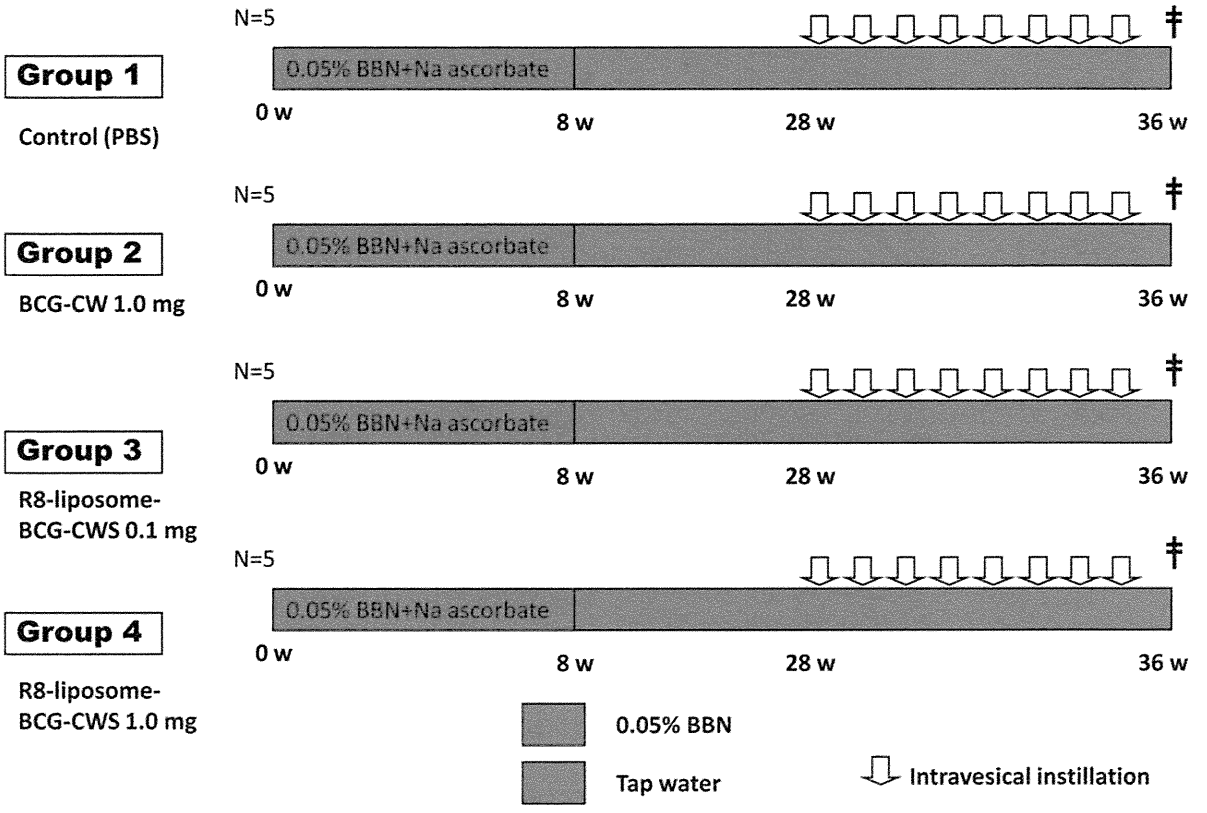


Figure 1. *Treatment protocol: 20 rats were divided into four groups.*

lipids of BCG (18), which involve inflammation and innate and acquired immune responses. However, BCG-CWS itself has difficulty associating with the bladder cancer cells due to its highly hydrophobic properties (19).

We previously reported that R8-liposome-BCG-CW completely inhibited the growth of MBT-2 tumors in C3H/HeN mice, whereas BCG-CW alone did not. In addition, animals vaccinated with a mixture of MBT-2 cells and R8-liposome-BCG-CW showed significant inhibition of the growth of R8-liposome-BCG-CW pretreated MBT-2 cells (11). This suggests that bladder cancer cells, which usually multiply under the condition of immune tolerance from the host, can be recognized through the presence of BCG-related molecules in cancer cells. The aim of the present study was to develop a non-live bacterial agent using a BCG-CWS, which consisted mainly of essential molecules, to induce immune responses (20-22).

The carcinogen BBN was used to induce urinary bladder tumors in the present study (14). Oral administration of BBN dependably induces urinary bladder tumors in a short time. In the rodent model of BBN-induced bladder carcinogenesis, the concentration of the carcinogen and duration of treatment determine the neoplasia in the urinary bladder. Nakanishi *et al*. stated that the lesions observed in the urinary bladder mucosa are classified as simple hyperplasia, PN hyperplasia, or papilloma (17), but cancerous cells were not found in any rat in their experiment (17). Thus, sodium ascorbate was administered to promote urinary bladder carcinogenesis (16). Administration of sodium ascorbate significantly increased the incidence and number of PN hyperplasias. There have been many reports on preneoplastic lesions in rat urinary bladder carcinogenesis (23), and there is strong evidence of correlation between PN hyperplasia and cancer of the urinary bladder. This finding could be related to the induction of cancer in rat urinary bladder by high levels of sodium ascorbate.

The results of the present study showed that both BCG-CW and R8-liposome-BCG-CWS (1.0 mg) inhibited rat bladder carcinogenesis induced by BBN administration. Both drugs, except for low-dose R8-liposome-BCG-CWS (0.1 mg), reduced the incidence of carcinoma and the mean number of tumors to approximately less than half of those of the control group. The formation of PN hyperplasia is thought to be a precursor to transitional cell carcinoma, and both drugs also inhibited PN hyperplasia.

Although the precise mechanism of the chemotherapeutic effect of R8-liposome-BCG-CWS has not been determined,