Cancer Chemother Pharmacol (2008) 61:973–978
DOI 10.1007/s00280-007-0553-1

ORIGINAL ARTICLE

New potential therapy for orthotopic bladder carcinoma by combining HVJ envelope with doxorubicin

Hirokazu Kawano · Shintaro Komaba · Tsugiko Yamasaki · Mitsuyo Maeda · Yoshimitsu Kimura · Akito Maeda · Yasufumi Kaneda

Received: 20 March 2007 / Accepted: 10 June 2007 / Published online: 26 July 2007
© Springer-Verlag 2007

Abstract
Purpose To establish a new therapeutic method to treat bladder carcinoma, we investigated the therapeutic potential of doxorubicin hydrochloride (DXR) combined with hemagglutinating virus of Japan-envelope vector (HVJ-E) in an orthotropic mouse bladder cancer model.
Methods DXR and/or HVJ-E were instilled into the bladder after implantation of MB49 cells. Antitumor effects of combination therapy were evaluated by histological analysis of the bladder on day 14 after tumor implantation. The survival rate of MB49-disseminated mice was examined for 60 days after single or double administration of DXR alone or DXR/HVJ-E. The surviving mice were re-challenged with intravesical injection of MB49 cells, and the bladder was observed after 3 weeks.
Results Combined intravesical instillation of HVJ-E and DXR resulted in a significantly higher rate of tumor-free mice (11/21) compared with mice treated using DXR alone (3/19, $P < 0.05$). Median survival was >60 days for intravesical instillation of HVJ-E and DXR, compared with the 29 days for DXR instillation alone ($P < 0.05$). After combination therapy, surviving mice formed no tumors in the bladder following intravesical re-instillation of MB49.
Conclusions HVJ-E increased antitumor effects in combination with chemotherapeutic agent (DXR). Antitumor immunity appeared to be enhanced using HVJ-E.

Keywords Bladder carcinoma · Chemotherapy · Viral envelope · Sendai virus

H. Kawano · S. Komaba · T. Yamasaki · Y. Kimura · A. Maeda
Saito Institute for Drug Discovery,
Research and Development, Genomidea Inc.,
Ibaraki, Osaka, Japan

H. Kawano · S. Komaba · Y. Kaneda (✉)
The Division of Gene Therapy Science,
Graduate School of Medicine, Osaka University,
2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: kaneday@gts.med.osaka-u.ac.jp

M. Maeda
Department of Anatomy and Neurobiology,
Osaka City University, Graduate School of Medicine,
Osaka, Japan

Introduction

Superficial bladder cancer is usually treated by transurethral resection of bladder tumor (TURBT), but a relatively high rate of recurrence and risk of progression to invasive cancer represent important problems [8]. The current treatment consists of TURBT for visible tumors, followed by intravesical chemotherapy to reduce disease recurrence and progression [1]. Until now, doxorubicin hydrochloride (DXR) and mitomycin C have been frequently used for this purpose [3, 5]. However, these drugs used intravesically in clinical trials on large case series have not proven capable of significantly reducing disease progression or improving overall survival [4, 16].

Intravesical Bacillus Calmette Guerin (BCG) is currently considered the most effective treatment against superficial bladder carcinoma [21]. BCG is generally considered to be an immunological agent, although the detailed mechanisms of action remain unclear. Indeed, while BCG therapy is the most successful example of immunotherapy to date, instillation of live BCG occasionally causes serious adverse effects [17]. Furthermore, administration of BCG is not effective for all patients, with approximately one-third of patients failing to respond [22], and combination with other anticancer drugs is not possible since BCG itself is a live

bacillus. Research into new drugs or drug combinations for bladder carcinoma has thus attracted a great deal of interest.

Hemagglutinating virus of Japan-envelope vector (HVJ-E) is a non-viral vector derived from inactivated Sendai virus [12]. Since the viral genome is inactivated, no replication or viral gene expression can occur in the host cell transfected with HVJ-E vector. HVJ-E is able to deliver plasmid DNA, short interfering RNA, proteins and drugs to various animal cells and tissues [13]. Moreover, HVJ-E itself can induce antitumor immunity by both enhancing cytotoxic T cells against cancers and inhibiting regulatory T cells [15]. Intraperitoneal injection of HVJ-E-incorporated bleomycin has thus been used to both eradicate tumors and generate antitumor immunity [19]. The present study investigated the therapeutic potential of HVJ-E combined with DXR against mouse bladder carcinoma.

Materials and methods

Animals

Six-week old female specific pathogen-free C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were used. All animals were housed in cages containing five animals and kept on a daily 12-h cycle of light and dark. Mice were provided with ad libitum access to water and standard CE-2 diet (Japan CLEA, Tokyo, Japan).

Tumor cells and tumor implantation

The MB49 murine transitional cell carcinoma cell line was obtained from Dr. Tim Ratliff (University of Washington University, St. Louis, MO, USA). Human bladder cancer cell line 5637 was purchased from Dainippon Sumitomo Pharma, Osaka, Japan. These cell line were maintained in RPMI1640 medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA) and 2 mM L-glutamine at 37°C and 5% CO_2. The MBT-2 murine bladder carcinoma cell line was purchased from Health Science Research Resources Bank, Osaka, Japan and maintained in MEM (Sigma-Aldrich Japan) supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C. For intravesical implantation of MB49 cells, C57BL/6 mice were anesthetized by intramuscular injection of a 9:1 ketamine (Sankyo, Tokyo, Japan)/xylazine (Bayer HealthCare, Osaka, Japan) mixture. The bladder was catheterized via the urethra using a 24-gauge plastic intravenous cannula (Nippon Becton Dickinson, Tokyo, Japan). Next, a 0.1-ml sample of a single-cell suspension containing 1×10^6 MB49 tumor cells in PBS with calcium, magnesium chloride and 1% BSA (Sigma) was instilled via cannula. Two mice out of 34 did not have tumors 2 weeks after the intravesical instillation of MB49 cells. The rate of tumor occurrence was ~94% in our experiment.

Preparation and intravesical injection of HVJ-E, DXR or DXR/HVJ-E

Adriacin Injection was purchased from Kyowa Hakko Chemical, Tokyo, Japan. DXR was prepared at 1 mg/ml by dissolving Adriacin injection in saline (Otsuka Pharm, Tokyo, Japan). Inactivated and purified HVJ-E was obtained from GenomIdea, Osaka, Japan. Five hundred hemagglutinating units (HAU) of HVJ-E was centrifuged at 15,000×*g* for 15 min at 4°C. The suspension was then removed and mixed with 100 µl of saline (HVJ-E) or 1 mg/ml of DXR (DXR/HVJ-E). DXR/HVJ-E was the mixture of doxorubicin and empty HVJ-E. On day 2 after injection of bladder carcinoma cells, intravesical injections of HVJ-E, DXR or DXR/HVJ-E were administered using the catheter described above.

Survival assay

Two hundred HAU of HVJ-E was centrifuged at 15,000×*g* for 15 min at 4°C. The suspension was then removed and mixed with 100 µl of 1 mg/ml of DXR (DXR/HVJ-E). On days 2 and 5 after injection of bladder carcinoma cells, 100 µl of 1 mg/ml of DXR or DXR/HVJ-E was injected intravesically using the catheter described above (total 400HAU). The mice were allowed to die because it was very difficult to judge the condition of the mice from their outlook after tumor inoculation. This experiment was performed according to the guidelines of GenomIdea company based on the guidelines of the Animal Committee of Osaka University.

WST-8 assay

MB49, MBT-2, and 5637 cells (1×10^4 cells) were seeded onto 96-well plates in 100 µl of RPMI 1640/10% FBS medium overnight, then 10 µl of saline, HVJ-E (200 HAU/ml), DXR (0.1 mg/ml) or DXR/HVJ-E (0.1 mg/ml/200 HAU) were added. After culturing for 48 h at 37°C in 5% CO_2, 10 µl of WST-8 cell count reagent, (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt dissolved in 1-Methoxy-5-methylphenazinium methylsulfate and NaCl; WAKO, Osaka, Japan) was added and further cultured for 1 h at 37°C in 5% CO_2. Absorbance was measured at 450 and 650 nm [20].

Histological confirmation

Mice were sacrificed on day 14 after implantation. Bladders were then removed, fixed with formalin and embedded in paraffin. Hematoxylin and eosin (HE) staining was performed on 4-µm sections. Sections were inspected for

tumor incidence and histological stage based on tumor size and localization. Bladder lesions were classified according to the classification of the General Rules for Clinical and Pathological Studies on Bladder Cancer [2].

Statistical analysis

Data were analyzed using the χ^2-test for histopathological results. Survival was compared using Kaplan-Meier methods and the generalized Wilcoxon test. Values of $P < 0.05$ were considered statistically significant. Kyplot 4.0 software (KyensLab, Tokyo, Japan) was used for all analyses.

Results

Cell proliferation assay in vitro

We first examined the effect of HVJ-E on growth rate of tumor cells in culture. Saline or HVJ-E alone was incubated with MB49 cells for 2 days. These conditions were not toxic against bladder tumor cells, and >80% of cells survived. DXR alone was highly effective at 0.01 mg/ml, killing >90% of MB49 cells, >90% of MBT-2 cells, and about 80% of 5637 cells compared with control cells in culture. Cytotoxic effects were also observed with the addition of DXR/HVJ-E, but there was no significant difference in cytotoxity between DXR/HVJ-E and DXR alone (Fig. 1). Addition of HVJ-E alone displayed no toxicity against bladder cancer cells in vitro.

Histopathological examination of the bladder

We next examined in vivo antitumor effects of HVJ-E, DXR, and DXR/HVJ-E. Mice were instilled with saline, HVJ-E, DXR or DXR/HVJ-E at 48 h after implantation of 1×10^5 MB49 cells into the bladder. After 14 days, massive tumors comprising MB49 cells were observed in eight mice instilled with saline or HVJ-E alone (Fig. 2a, b). DXR-treated mice also displayed large tumors, although three mice exhibited no visible tumor in the bladder (Fig. 2c). In contrast, tumor formation in the bladder appeared to be inhibited in mice with DXR/HVJ-E instillation (Fig. 2d). A few mice showed infiltration of lymphocytic inflammatory cells or edema under the mucosa, but no significant damage was observed in bladder tissue following DXR/HVJ-E treatment.

Sections of mouse bladder were classified by histological stage (Table 1) [2]. Low-stage superficial carcinoma (pTis/pTa) was not seen in any groups. Stage pT1 and pT2< were seen in controls (8/9), HVJ-E (8/9), DXR (16/19), and DXR/HVJ-E (10/21). Moreover, approximately half of the mice instilled with DXR/HVJ-E (11/21) remained tumor-free (pT0) in the bladder by day 14 after implantation. These data show that instillation of DXR/HVJ-E could strongly suppress tumor growth compared with treatment using DXR alone ($P < 0.05$).

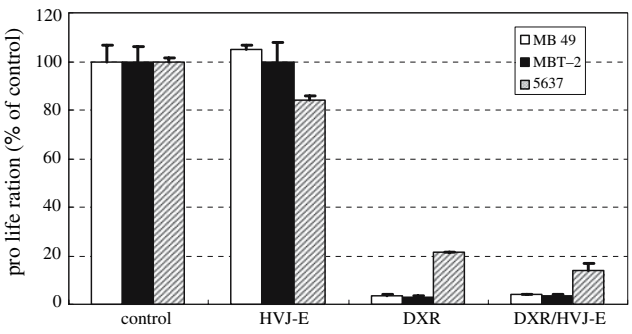

Fig. 1 Cell proliferation by WST-8 assay. Bladder cancer cell lines MB49, MBT-2 or 5637 were incubated with saline (control), HVJ-E (2 HAU), DXR (1 μg) or DXR/HVJ-E (1 μg/2 HAU) for 2 days. Mean ± SD ($n = 3$) are shown for each group. Three replicate plates were used for the in vitro assays and the experiment was repeated twice. As the results of the two independent experiments were almost same, one representative result was presented

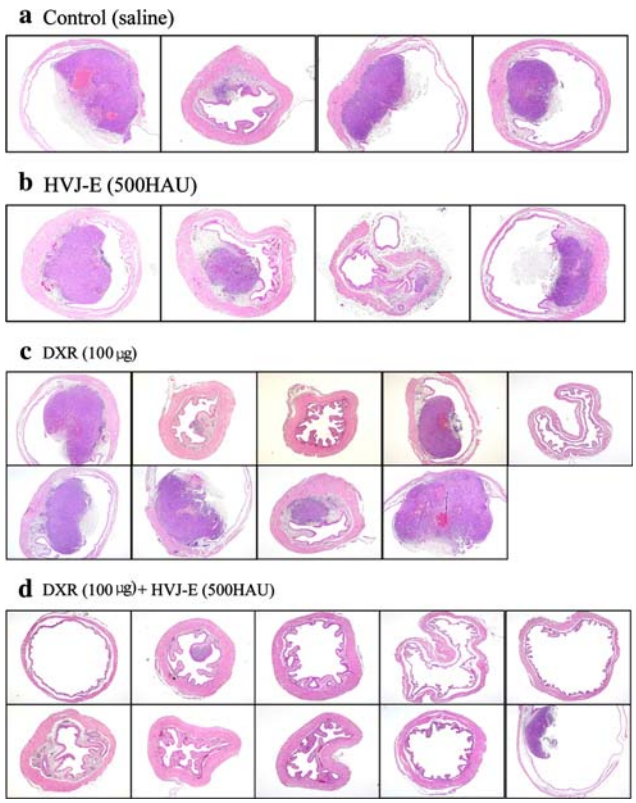

Fig. 2 HE staining of MB49-implanted bladders. On day 2 after intravesical implantation of MB49 cells, saline (**a**), 500 HAU of HVJ-E (**b**), 100 μg of DXR (**c**) or 100 μg DXR/500 HAU HVJ-E (**d**) was instilled once into the bladder for 10 min. On day 14, bladders were removed and stained using HE

Table 1 Histological stage of bladder samples from Fig. 2

	N	pT0	pTis/pTa	pT1	pT2<
MB49	9	1	0	3	5
MB49 + HVJ-E	9	1	0	6	2
MB49 + DXR	19	3	0	7	9*
MB49 + DXR/HVJ-E	21	11	0	8	2*

All the bladder samples were subjected to the preparation of ten sections with the equal distance followed by HE staining

N number of samples, *pT0* no tumor; *pTis/pTa, pT1* superficial carcinoma, *pT1* infiltration under the mucosa, not under the muscle layer, and *pT2<* infiltration under the muscle layer

*Histological stage for mice with instillation of DXR/HVJ-E differed significantly from mice receiving DXR, $P < 0.05$, χ^2 -test

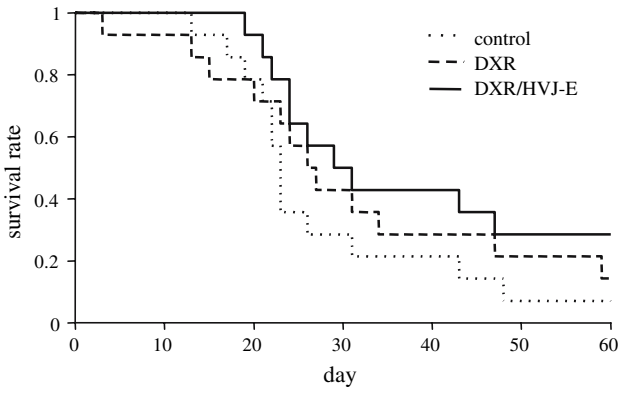

Fig. 3 Survival rate of MB49-implanted mice after single treatment with saline, 100 μg of DXR, or 100 μg of DXR/200 HAU HVJ-E. Median survival times were 19 days for saline, 24 days for DXR and 30.5 days for DXR/HVJ-E ($n = 7$ for each group). No significant differences in survival were seen between DXR and DXR/HVJ-E groups

Effect of DXR or DXR/HVJ-E instillation on survival

We examined survival rates of MB49-disseminated mice after single administration of DXR or DXR/HVJ-E. Mice were intravesically implanted on day 0 with 1×10^5 MB49 cells, then instilled with saline, DXR or DXR/HVJ-E into the bladder on day 2. Mice treated with saline showed rapid tumor growth and median overall survival was 19 days (Fig. 3). Instillation of HVJ-E resulted in a similar survival rate to that seen in controls (data not shown). Conversely, median survival was 24 days for mice treated with DXR and 30.5 days for mice treated using DXR/HVJ-E (control versus DXR, $P = 0.46$; control versus DXR/HVJ-E, $P = 0.07$). No significant difference in median survival was seen between mice instilled with DXR and DXR/HVJ-E ($P = 0.36$).

We next attempted to enhance the antitumor effect of DXR/HVJ-E by consecutive administration of DXR/HVJ-E into the bladder. Mice implanted on day 0 with MB49 received two instillations of saline, DXR or DXR/HVJ-E into the bladder, on days 2 and 5. Median overall survival of mice treated using saline was 20 days, similar to that seen with single instillation (Fig. 4). Median survival of mice treated with double administration of DXR was 29 days, only 5 days longer than survival following single administration of DXR. However, median survival of mice treated twice with DXR/HVJ-E was >60 days. An exact median survival could not be calculated because more than 50% of these mice were not dead as of the end of the study following second administration of DXR/HVJ-E. Instillation of DXR/HVJ-E twice thus significantly prolonged median survival compared with DXR treatment ($P < 0.05$).

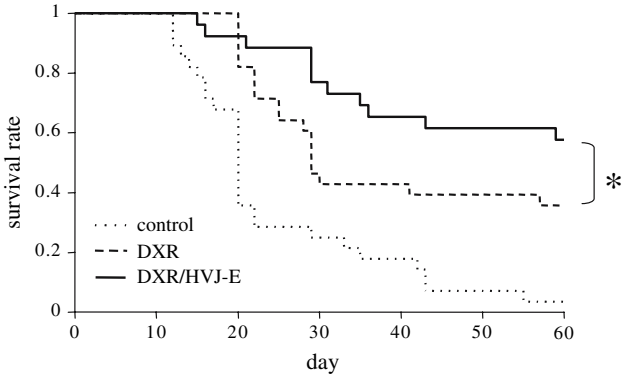

Fig. 4 Survival rate of MB49-implanted mice after consecutive treatment with saline ($n = 28$), 100 μg of DXR ($n = 28$), or 100 μg of DXR/200HAU HVJ-E ($n = 26$). The DXR/HVJ-E group showed significantly longer survival than the DXR group. *$P < 0.05$ DXR versus DXR/HVJ-E, Generalized Wilcoxon test

When surviving mice were re-challenged with intravesical injection of MB49, none of the ten mice treated with DXR/HVJ-E formed tumors by 3 weeks after re-challenge, while two of six mice treated with DXR alone macroscopically formed large tumors in the bladder (Fig. 5).

Discussion

Treatment of superficial bladder cancer is aimed at eradicating existing disease and preventing tumor recurrence, muscle invasion and metastasis. However, clinical use of intravesical instillation for anticancer drugs has not yet resulted in significant improvements to overall survival. Despite the fact that intravesical BCG instillation offers high efficacy, its use in clinical practice is limited due to the potential adverse effects [23]. Furthermore, one-third of patients treated with BCG develop recurrent bladder cancer [10]. Novel approaches for eradicating bladder carcinoma with minimum toxicity have thus been preferred. The present study investigated the possibility of treating bladder

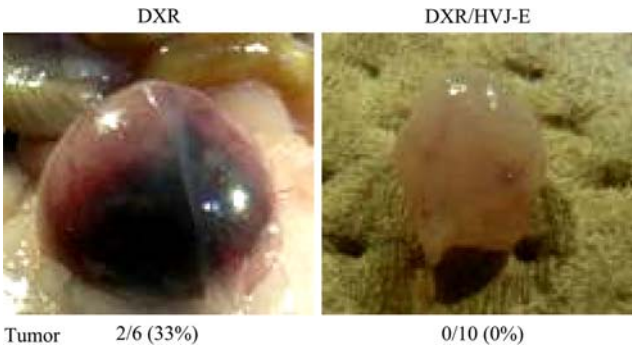

Fig. 5 Re-challenge of surviving mice with intravesical injection of MB49 cells. Surviving MB49 implanted-mice treated with DXR or DXR/HVJ-E were intravesically re-instilled with 1×10^6 cells

carcinoma using a combination of DXR and HVJ-E in the MB49 murine bladder cancer model.

HVJ-E is unable to replicate in cells because the genome is destroyed by UV irradiation and/or β-propiolactone treatment [12]. HVJ-E treatment would thus seem likely to be less toxic to cells than instillation of BCG using live bacillus. WST-8 assay showed that HVJ-E was non-toxic for murine and human bladder cancer cell lines (Fig. 1), and no significant tissue damage was found in bladder treated with HVJ-E (data not shown).

To test the therapeutic efficacy of HVJ-E, we established an orthotopic bladder carcinoma model as described previously [7]. However, compared with the conditions of previous reports [7], our cancer model seems more aggressive. The number of MB49 cells inoculated into each bladder was tenfold more in our model (1,000,000 cells) than in the previous model (100,000 cells). We started therapeutic treatments on day 2 after tumor inoculation, although previous experiment was conducted 1 day after tumor implantation. DXR/HVJ-E was intravesically instilled up to two times (either on day 2 or on days 2 and 5) and mice were observed for 60 days. However, in the previous study, BCG was injected 4 times (days 1, 8, 15, and 22) and the experiment was terminated at day 28 due to rapid tumor growth. Considering that mouse survival rate was 60% using two instillations of DXR/HVJ-E in our cancer model, the therapeutic potential of DXR/HVJ-E may be higher than BCG.

Histopathological examination revealed that there was no significant difference in tumor growth by DXR treatment compared with either saline or HVJ-E treatment. Conversely, tumors in the DXR/HVJ-E treatment group were obviously smaller than those of other groups, and many mice displayed no tumors at all (Fig. 2; Table 1). However, no significant difference in mouse survival was seen between DXR and DXR/HVJ-E groups. This suggests that single administration of DXR/HVJ-E was insufficient to completely eradicate tumor cells and permitted tumor recurrence. When DXR/HVJ-E was administered into the bladder twice, more than half of the mice survived for >2 months, while half of the mice treated twice using DXR alone died within 1 month (Fig. 4). Double administration of DXR alone extended median survival by only 5 days compared with single administration. This suggests that consecutive administration of DXR/HVJ-E might induce other anticancer effects in addition to direct chemotherapeutic killing effects. When the surviving mice were re-challenged with intravesically injected MB49, all ten mice treated with DXR/HVJ-E remained tumor-free as of 3 weeks after re-challenge, while two of six mice treated with DXR alone formed large tumors in the bladder (Fig. 5). These results suggest that consecutive instillations of DXR/HVJ-E may enhance effective antitumor immunity in the bladder more than DXR only.

HVJ-E is known to induce maturation of dendritic cells in a Toll-like receptor-independent manner [11]. We recently reported that HVJ-E can induce cytotoxic T cells against cancers and inhibit regulatory T cells by IL-6 expression in dendritic cells [15]. These facts support the induction of anticancer immunity in bladder by consecutive DXR/HVJ-E treatments.

Intravesical instillation of BCG has been regarded as highly effective for superficial bladder tumors [23]. BCG is believed to activate local immunological reactions requiring the presence of CD4 and CD8 T cell subsets [24]. Intravesical BCG immunotherapy increases cytokines such as interleukin 1, 2, and 6, TNFα and IFNγ in urine [6]. These immunological responses are mediated mainly by Toll-like receptor (TLR)-2 and 4 [9, 25]. TLR-9 may also be involved in the response [26]. On the other hand, since antitumor immunity by HVJ-E is independent of current TLRs [18], the mechanism of antitumor activity for HVJ-E is considered to be quite different from that of BCG. DXR/HVJ-E may thus offer effective therapy for non-responders to BCG treatment.

We are now testing the effect of HVJ-E or DXR/HVJ-E on tumor initiation and promotion using chemical-substance, *N*-butyl-*N*(4-hydroxybutyl) nitrosamine, induced bladder tumor model in Fischer 344 rats [14] because no effective treatment has been established for this tumor model. Toxicity of HVJ-E or DXR/HVJ-E after consecutive administration into the bladder will be examined both regionally in the bladder and systemically in other organs. Systemic distribution of radio-labeled HVJ-E after intravesical administration will be also investigated. We have succeeded in the large-scale production of HVJ in human cells, not in chick egg, using completely animal product-free medium, and also established purification technology of HVJ-E using three-different column system in GMP-grade facility [13]. Clinical grade HVJ-E is now ready for use. Based on those preclinical studies, we are going to start Phase-I study of DXR/HVJ-E treatment in bladder cancer

patients resistant to other therapeutics. There are still many hurdles to be overcome before the clinical use of DXR/HVJ-E to bladder carcinoma.

References

1. Herr HW (1987) Current medical therapy for urologic disease. Urol Clin North Am 14:247–417
2. Association JU (2001) General rules for clinical and pathological studies on bladder cancer. Kanehara
3. Chai M, Wientjes MG, Badalament RA, Burgers JK, Au JL (1994) Pharmacokinetics of intravesical doxorubicin in superficial bladder cancer patients. J Urol 152:374–378
4. Crawford ED (2002) Intravesical therapy for superficial cancer: need for more options. J Clin Oncol 20:3185–3186
5. Dalton JT, Wientjes MG, Badalament RA, Drago JR, Au JL (1991) Pharmacokinetics of intravesical mitomycin C in superficial bladder cancer patients. Cancer Res 51:5144–5152
6. De Boer EC, De Jong WH, Steerenberg PA, Aarden LA, Tetteroo E, De Groot ER, Van der Meijden AP, Vegt PD, Debruyne FM, Ruitenberg EJ (1992) Induction of urinary interleukin-1 (IL-1), IL-2, IL-6, and tumour necrosis factor during intravesical immunotherapy with bacillus Calmette-Guerin in superficial bladder cancer. Cancer Immunol Immunother 34:306–312
7. Gunther JH, Jurczok A, Wulf T, Brandau S, Deinert I, Jocham D, Bohle A (1999) Optimizing syngeneic orthotopic murine bladder cancer (MB49). Cancer Res 59:2834–2837
8. Hall RR, Parmar MK, Richards AB, Smith PH (1994) Proposal for changes in cystoscopic follow up of patients with bladder cancer and adjuvant intravesical chemotherapy. BMJ 308:257–260
9. Heldwein KA, Liang MD, Andresen TK, Thomas KE, Marty AM, Cuesta N, Vogel SN, Fenton MJ (2003) TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. J Leukoc Biol 74:277–286
10. Hudson MA, Herr HW (1995) Carcinoma in situ of the bladder. J Urol 153:564–572
11. Ito T, Amakawa R, Inaba M, Hori T, Ota M, Nakamura K, Takebayashi M, Miyaji M, Yoshimura T, Inaba K, Fukuhara S (2004) Plasmacytoid dendritic cells regulate Th cell responses through OX40 ligand and type I IFNs. J Immunol 172:4253–4259
12. Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N, Nakamura H, Morishita R, Kotani H (2002) Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. Mol Ther 6:219–226
13. Kaneda Y, Yamamoto S, Nakajima T (2005) Development of HVJ envelope vector and its application to gene therapy. Adv Genet 53:307–332
14. King EB, Vanderlaan M, Jensen RH, Kromhout LK, Hoffman JW (1984) Morphologic changes in rat urothelial cells during carcinogenesis: I. Histologic and cytologic changes. Cytometry 5:447–453
15. Kurooka M, Kaneda Y (2007) Inactivated Sendai virus particles eradicate tumors by inducing immune responses through blocking regulatory T cells. Cancer Res 67:227–236
16. Lamm DL, Riggs DR, Traynelis CL, Nseyo UO (1995) Apparent failure of current intravesical chemotherapy prophylaxis to influence the long-term course of superficial transitional cell carcinoma of the bladder. J Urol 153:1444–1450
17. Lamm DL, van der Meijden PM, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A, Debruyne FM (1992) Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. J Urol 147:596–600
18. Lopez CB, Moltedo B, Alexopoulou L, Bonifaz L, Flavell RA, Moran TM (2004) TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. J Immunol 173:6882–6889
19. Mima H, Yamamoto S, Ito M, Tomoshige R, Tabata Y, Tamai K, Kaneda Y (2006) Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector. Mol Cancer Ther 5:1021–1028
20. Miyamoto T, Min W, Lillehoj HS (2002) Lymphocyte proliferation response during Eimeria tenella infection assessed by a new, reliable, nonradioactive colorimetric assay. Avian Dis 46:10–16
21. Morales A, Eidinger D, Bruce AW (1976) Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J Urol 116:180–183
22. Nawrocki S, Skacel T, Skoneczna I (2002) Pharmacotherapy of bladder cancer—practice and prospects. Expert Opin Pharmacother 3:671–679
23. Nseyo UO, Lamm DL (1997) Immunotherapy of bladder cancer. Semin Surg Oncol 13:342–349
24. Ratliff TL, Ritchey JK, Yuan JJ, Andriole GL, Catalona WJ (1993) T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. J Urol 150:1018–1023
25. Uehori J, Matsumoto M, Tsuji S, Akazawa T, Takeuchi O, Akira S, Kawata T, Azuma I, Toyoshima K, Seya T (2003) Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by Mycobacterium bovis bacillus Calmette-Guerin peptidoglycan. Infect Immun 71:4238–4249
26. von Meyenn F, Schaefer M, Weighardt H, Bauer S, Kirschning CJ, Wagner H, Sparwasser T (2006) Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. Immunobiology 211:557–565