

ORIGINAL ARTICLE

Tetracycline-inducible promoter-based conditionally replicative adenoviruses for the control of viral replication

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The use of conditionally replicative adenoviruses (CRAds) as a promising strategy for cancer gene therapy has been developed to overcome inefficient transduction of solid tumor masses by replication-deficient adenoviruses. Many modifications have been made to CRAds to enlarge tropism, increase selectivity and lytic ability, and improve safety. However, safety is still a concern in the context of future clinical application of CRAds. Particularly, after injection into the body, viral replication cannot be controlled externally. Therefore, we constructed a novel CRAd using a tetracycline-inducible promoter system to realize external pharmacological control of its replication. The effect of this CRAd *in vitro* was measured at the levels of viral DNA replication, cell death and progeny production. We showed that CRAd replication was tightly controlled by the presence or absence of doxycycline (Dox). Moreover, this system showed a significant gene expression *in vivo*, in which the viral replication was controlled by the oral administration of Dox. This strategy may help improve the safety of cancer gene therapy.

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Keywords: conditionally replicative adenovirus; tetracycline; inducible promoter

Introduction

Viral oncolysis or virotherapy represents a novel promising strategy for cancer gene therapy. Various candidate viruses, such as adenovirus,^{1–3} herpes simplex virus,^{4,5} reovirus,^{6,7} influenza virus,^{8,9} Newcastle disease virus,^{10,11} poliovirus,^{12,13} vaccinia virus^{14,15} and vesicular stomatitis virus,¹⁶ have been used to replicate selectively in malignant tissue. Among them, a conditionally replicative adenovirus (CRAd) is one of the most attractive candidates having the critical properties required for viral oncolysis.^{17,18} CRAds initially infect tumor cells and undergo replication, followed by oncolysis and then subsequent release of the virus progeny. This replication cycle allows dramatic local amplification of the input dose. In addition, their life cycle is lytic, and after completing a replication cycle they kill the host tumor cells during viral release. In theory, a CRAd would replicate until all the tumor cells allowing the replication are lysed.¹⁹ CRAd released from the tumor cells might then disseminate and infect distant metastases. Furthermore, eradication of systemic disease might be enhanced by the immune response directed against infected tumor

cells.²⁰ However, there are some problems to address regarding viral therapy. One of these is the absence of a regulatory system for viral replication. As cancer-specific promoters integrated into the CRAd genome only activate the E1 gene expression positively, we cannot suppress viral self-replication. Excessive viral production and release into the blood causes intense immune reaction with antibodies, leading to severe damages to many organs. Another problem is the presence of the anti-adenovirus antibody as an immune barrier. Only a small part of the injected virus can reach a tumor through blood circulation because of fatal interaction with anti-adenovirus antibodies. Pre-existing or induced anti-adenovirus antibodies trap the virus virions and neutralize their infectious ability. To overcome such physical barrier, a recent paper showed the usefulness of mesenchymal progenitor cells as a virus carrier when loaded with a replicative adenovirus.²¹ A replicative adenovirus seems to reach a tumor through the cell-vehicle system, slipping through the antibody barrier in the blood. However, it may be difficult for the replicative virus to destroy and escape from vehicle cells at an optimal timing because of an uncontrollable promoter. To overcome such problems, we developed a replication-controllable adenovirus based on a pharmacological inducible system as shown in Figure 1. The tetracycline-inducible gene expression system developed by Gossen and Bujard^{22,23} has proven useful for inducing or repressing, at will, the expression of a particular gene in mammalian cells. This system relies on the presence or absence of tetracycline or a commonly

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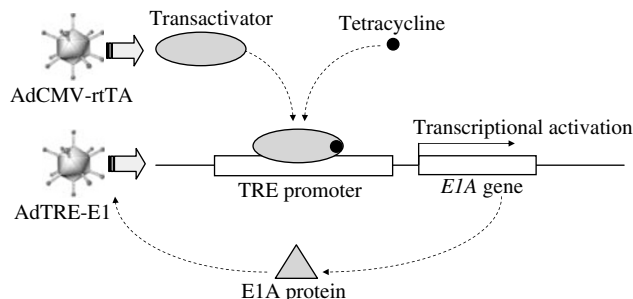


Figure 1 Scheme of tetracycline-inducible CRAd system. The replication of the CRAd and AdTRE-E1 is under the control of a tetracycline-inducible promoter containing the tetracycline-responsive element (TRE). The recombinant transactivator protein is supplied from AdCMV-rtTA. After the conformational change with tetracycline, the transactivator protein provides binding activity to TRE, and activates the promoter transcriptionally. Then, the expressed E1A protein triggers the replications of the AdTRE-E1 and AdCMV-rtTA genomes. Without tetracycline, the transactivator protein remains inactive and the viruses cannot replicate. In this system, tetracycline functions as a switch for viral replication.

used analog, doxycycline (Dox), to control gene expression. As the gene expression is highly specific and tightly controlled, the tetracycline-inducible gene expression system has become widely used. In this study, we constructed a novel rtTA-CRAD system and showed that it can control CRAd replication *in vitro* and *in vivo*. Thus, we can improve CRAd safety to a great extent in the context of future virotherapy.

Materials and methods

Cell culture

The following cells were used in the experiment: human embryonic kidney 293 cells and 911 cells, human fibroblast WI38 cells, human cervical carcinoma HeLa cells and human lung adenocarcinoma A549 cells. The cells were maintained in appropriate medium, and preserved in a humidified incubator with 5% CO₂ at 37 °C. The medium used was supplemented with 10% fetal bovine serum (Sigma-Aldrich Corporation, St Louis, MO) and 1% penicillin–streptomycin (Invitrogen Corporation, Carlsbad, CA). *In vitro* gene transfer into cells was carried out by incubation with an adenoviral vector in medium containing 2% fetal bovine serum (Sigma-Aldrich) and 1% penicillin–streptomycin (Invitrogen Corporation) in a humidified incubator with 5% CO₂ at 37 °C for 3 h with gentle agitation per hour, followed by the addition of the complete medium.

Construction of adenoviral plasmid

A fragment of the 0.45-kb tetracycline-responsive element (TRE) was removed from the Pxp2-TRE plasmid by digestion with *Eco*RI, and then the plasmid was digested with *Xho*I after blunting. The fragment was ligated to the plasmid pShuttleE1²⁴ producing pShuttleTRE-E1. The pShuttleluc1 plasmid was digested with *Hind*III initially,

and then digested with *Xho*I after blunting. Thereafter, the TRE fragment was ligated to the pShuttleluc1 to construct the pShuttleTRE-luc1. Each shuttle plasmid was co-transfected to BJ5183 with the backbone plasmid pAdEasy (Stratagene, La Jolla, CA) by electroporation.²⁵ After homologous recombination, the resulting adenoviral plasmids were designated as pAdTRE-E1 and pAdTRE-luc1.

Production of recombinant adenoviruses

pAdTRE-E1 and pAdTRE-luc1 were transfected to 911 cells using the lipofection method, as described earlier.²⁵ The resulting viruses, AdTRE-E1 and AdTRE-luc1, showed no regeneration of the wild-type adenovirus, as confirmed by PCR. The sequences of the PCR primers were as follows: Ad5ITR-sense: 5'-AAAAAATGACGTAACGGTTAAAGTCCA; E1-antisense: 5'-CACGTCAGCTGACTATAATAAT; luciferase-antisense: 5'-AAGTGATGTCCACCTCGATATGTGCAT. AdCMV-rtTA expressing the transcriptional activator on TRE was obtained from BD Biosciences (San Jose, CA). AdTRE-E1, AdTRE-luc1, Ad5CMV-rtTA and the negative control virus AdCMV-luc1 were propagated in 293 cells and purified by two rounds of cesium chloride density ultra-centrifugation. The titer of the viruses was obtained by determining the tissue culture infectious dose 50.

Luciferase activity assay

HeLa cells (1×10^6) were infected with AdCMV-luc1, AdTRE-luc1, AdCMV-rtTA, or co-infected with AdCMV-rtTA and AdTRE-luc1 at multiplicity of infection (MOI) 10. After 3 h of infection, the medium was replaced with fresh complete medium with or without $1 \mu\text{g ml}^{-1}$ Dox (Wako Pure Chemical Industries, Osaka, Japan), and incubated for another 48 h. Infected cells were collected and luciferase activity was measured using the luciferase assay system with reporter lysis buffer (Promega Corporation, Madison, WI) according to the manufacturer's protocol. In *in vivo* experiment, the resected organs were immediately frozen in liquid nitrogen. Frozen tissues ground to a fine powder were lysed using a tissue lysis buffer (Promega), and then luciferase activity was determined using a luciferase assay kit (Promega). The luciferase activity was normalized by protein concentration in the tissue lysate.

Crystal violet cell viability assay

HeLa cells were plated at 3×10^5 cells per well in 24-well plates and incubated to allow cell attachment. Before infection, the medium was removed and cells were washed with physiological buffered saline. Then the cells were co-infected with AdTRE-E1 and AdCMV-rtTA at MOI 10 at a ratio of 1:1 with or without Dox. After 3 days of infection, the cells were carefully washed with physiological buffered saline, and then 1 ml of 1% crystal violet in 70% ethanol was added to each well to allow staining for 1 h, followed by washing with tap water until excess color was removed. The experiment was performed in triplicate wells.

Cell proliferation assay

HeLa cells were seeded at 1×10^4 cells per well in a 96-well flat-bottomed plate and incubated at 37°C overnight to allow them to attach. Then cells were co-infected with AdTRE-E1 and AdCMV-rtTA at MOI 10 at a ratio of 1:1 with or without Dox. The medium volume was 0.2 ml per well. After 3 days of infection, 22 μl Tetracolor One (Seikagaku Co., Tokyo, Japan) was added to cell cultures in each well and cultured for 2 h as described earlier.²⁶ The absorbance was measured with an enzyme-linked immunosorbent assay reader at 450 nm.

Progeny viral particle titration

HeLa cells (1×10^6) were seeded in 50-mm plates and allowed to attach overnight. The next day, the cells were co-infected with AdTRE-E1 and AdCMV-rtTA at MOI 10 and incubated without Dox. After 3 h of infection, the cells were trypsinized and replated in new 24-well plates to completely inactivate the remaining parental virus. Then, the cells were incubated for another 3 days with or without Dox. All experiments were performed in triplicate wells. Finally, the culture medium was collected and tissue culture infectious dose 50 was determined using 293 cells to confirm the titer of the progeny virus released into the medium.

Analysis of viral genome amplification

Viral DNA amplification was assessed as reported earlier.²⁷ HeLa cells were plated in a 12-well culture plate in triplicate at the density of 1×10^5 cells per well. After an overnight culture, the cells were co-infected with AdTRE-E1 and AdCMV-rtTA at MOI 10 and then incubated with or without Dox for 24 h. Viral DNA isolation was carried out using a blood DNA kit (Qiagen, Valencia, CA) after harvesting the cells. The viral DNA was eluted with 100 μl of elution buffer [10 mM Tris-Cl (pH 8.5)]. Eluted samples (1 μl) were analyzed by real-time PCR analysis to evaluate adenoviral E1 and E4 copy numbers using a LightCycler (Roche Applied Science, IN). Oligonucleotides corresponding to the sense strand of the Ad E4 region (5'-TGACACGCATACTCGGAGC TA-3': 34 885–34 905 nt), the antisense strand of the E4 region (5'-TTTGAGCAGCACCTTGCATT-3': 34 977–34 958 nt) and a probe (5'-CGCCGCCCATGCAA CAAGCTT-3': 34 930–34 951 nt) were synthesized and used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C , 20 s), annealing (55°C , 20 s) and extension (72°C , 30 s). The adenovirus backbone vector pTG3602 (Transgene, Strasbourg, France)²⁸ was used for making a standard curve for the Ad E4 DNA copy number. The E4 copy numbers were normalized by the β -actin DNA copy number.

Animals

Female nude mice (BalbC nu/nu), 6 weeks old, were used. The experiment was carried out under both the Guidelines for Animal Experiments of Kyushu University, and the Law and Notification of the Japanese government. The mice were housed four to six per cage and kept under

standard laboratory conditions, during which food and water were supplied *ad libitum*. Then the mice were allowed to acclimate themselves to the new environment after shipping for 1 week before treatment.

Tetracycline promoter-inducible gene activation in vivo

The WI38 cells (1×10^6) co-infected with AdCMV-rtTA, AdTRE-E1 and AdCMV-luci, respectively, at MOI 10 were transplanted into the peritoneal space in the nude mice. After transplantation, the mice were fed with drinking water containing 500 $\mu\text{g ml}^{-1}$ of Dox dissolved in 5% sucrose for 3 days. Then, the mice were killed and the organs, including liver, spleen, kidney, lung and mesenterium, were resected for luciferase assay as described above.

Statistical analysis

ANOVA was used to examine the significance of any differences between the two groups with a single independent variable. A *P*-value less than 0.05 was considered statistically significant. All data are presented as mean \pm s.e.m.

Results

Transcriptional activation of tetracycline-responsive promoter

We initially confirmed that the correct recombinant adenoviruses were constructed by PCR and restriction enzyme digestion analysis (data not shown). Next, we then confirmed the transcriptional activity of the tetracycline-responsive promoter by luciferase-activity assay. The luciferase activity of the HeLa cells infected with AdCMV-rtTA as a negative control virus was very low, whereas that of the cells infected with AdCMV-luci was extremely high. HeLa cells infected with AdTRE-luci showed a marginal luciferase activity probably due to the leakiness of the TRE promoter. On the other hand, HeLa cells infected with the relevant adenoviruses showed similar luciferase activities in the presence or absence of Dox in the culture medium. We found that higher luciferase activity was induced in HeLa cells co-infected with AdCMV-rtTA and AdTRE-luci with Dox than in those without Dox ($P < 0.05$), as shown in Figure 2. Similar data were obtained using A549 cells instead of HeLa cells (data not shown). From these data, it was confirmed that the tetracycline regulation system could be used in the context of the recombinant adenoviral genome.

Viral DNA replication

The replicated viral DNA was quantified as E1 and E4 copy numbers by real-time PCR. As shown in Figure 3, the AdTRE-E1 viral genome replicated in the HeLa cells in the presence of Dox, and the copy numbers of E1 and E4 were more than $1.0\text{E} + 7$, whereas those in the absence of Dox were $1.0\text{E} + 4.7$ and $1.0\text{E} + 4.6$, respectively. Importantly, the replication of the AdTRE-E1 genome was induced by the addition of Dox, and each copy

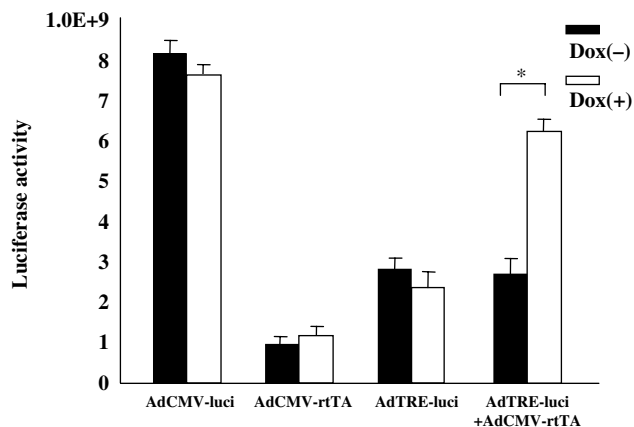


Figure 2 Promoter activities induced by the tetracycline-inducible system. HeLa cells (1×10^5) were infected with AdCMV-luci, AdCMV-rTA and AdTRE-luci at multiplicity of infection (MOI) 10 for 3 h. Then, the infected cells were incubated with (open square) or without (closed square) $1 \mu\text{g ml}^{-1}$ Dox for 48 h, and harvested and lysed in $100 \mu\text{l}$ of lysis buffer. Each lysate ($10 \mu\text{l}$) was used for luciferase assay. HeLa cells were also co-infected with AdCMV-rTA and AdTRE-luci at MOI 10, and the luciferase activities were measured in the same manner (* $P < 0.05$). Mean \pm s.e. of triplicate determination is shown.

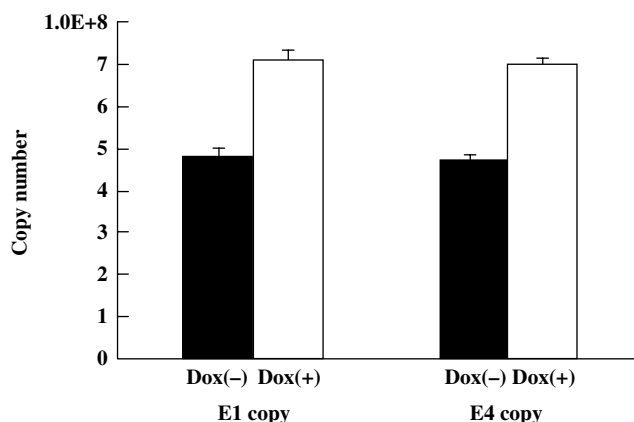


Figure 3 Quantification of replicated viral DNA 24 h after infection. HeLa cells (1×10^5) were infected with AdTRE-E1 and AdCMV-rTA at multiplicity of infection (MOI) 10 and then incubated with (open square) or without (closed square) Dox for 24 h. Viral DNA was isolated from the cells and analyzed by the real-time PCR quantification method to evaluate adenoviral E1 and E4 copy numbers, as described in Materials and methods. E1 and E4 copy numbers were normalized by the β -actin DNA copy number. Mean \pm s.e. of triplicate determination is shown.

number for E1 and E4 regions was 2.5-logs higher than that without Dox. These results indicate that the tetracycline-inducible promoter retains fidelity even in the replication-competent adenoviral context.

Progeny virus production

Viral replication ability was evaluated by the titration of the progeny virus virion released into the medium. Titration was performed 3 days after infection using the

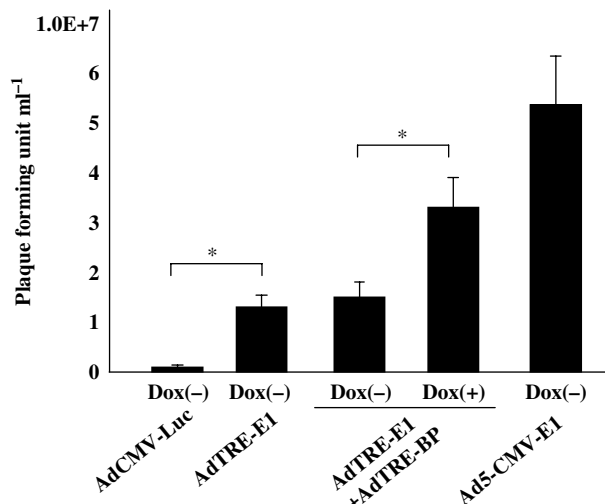


Figure 4 HeLa cells were infected with AdCMV-luci, AdTRE-E1 and AdCMV-E1 without Dox. These cells were also co-infected with AdCMV-rTA and AdTRE-E1 with or without Dox. At 3 days after infection, $50 \mu\text{l}$ of the culture medium was removed and subjected to plaque-forming unit assay. HeLa cells co-infected with AdCMV-rTA and AdTRE-E1 produced significantly larger amounts of progeny viruses ($P < 0.05$) with Dox. Mean \pm s.e. of triplicate determination is shown.

plaque-forming assay. As shown in Figure 4, the medium of the cells infected with AdCMV-luci (replication-incompetent virus), included a small number of adenoviruses. On the other hand, the cells infected with only AdTRE-E1 released a significantly higher amount of progeny virus than those infected with AdCMV-luci. AdTRE-E1 may express the E1 protein owing to the leakiness of the TRE promoter or enhancer function of the terminal repeat sequence of the adenoviral vector. When co-infected with Ad5CMV-rTA and Ad5TRE-E1, a similar result was obtained in the absence of Dox. However, the number of viral progeny in the medium of the cells co-infected with Ad5CMV-rTA and Ad5TRE-E1 with Dox was 16-fold higher than those without Dox. Although the number was lower than that of the cells infected with AdCMV-E1, the data suggested that the tetracycline-inducible promoter functions well and triggers virus replication and viral progeny production successfully.

Cell-killing effect

The cell-killing effect was analyzed by crystal violet staining of viable cells and Tetracolor One cell proliferation assay. The cytopathic effect was observed in the HeLa cells co-infected with AdCMV-rTA and AdTRE-E1 with Dox, but not in those without Dox (data not shown). This was confirmed by cell proliferation assay. There was a significant difference in cell viability between the cells co-infected with AdCMV-rTA and AdTRE-E1 with Dox and those without Dox ($P < 0.01$, Figure 5a). However, a mild cell-killing effect was observed in the cells co-infected with AdCMV-rTA and AdTRE-E1 with Dox compared with the uninfected intact cells. In the next

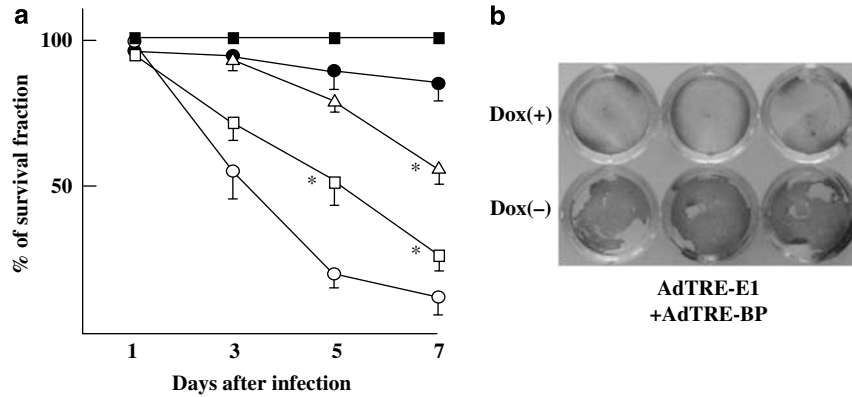


Figure 5 (a) HeLa cells were co-infected with AdCMV-rtTA and AdTRE-E1 at multiplicity of infection (MOI) 10, and incubated with (open circle) or without (closed circle) Dox. As a positive control, HeLa cells were also infected with AdCMV-E1 at MOI 10 (open square). The viability of the cells was evaluated by MTS assay, and the survival fraction was shown as percentage of the intact cells (closed square) at each point. After 3 days, the survival fraction of the cells infected with AdCMV-E1 decreased rapidly. When incubated with Dox, co-infection with AdCMV-rtTA and AdTRE-E1 also showed a strong cell-killing effect; however, the cell-killing effect was weak without Dox. At days 5 and 7, there was a significant difference in the cell-killing effect between the cells incubated with (open circle) or without (closed circle) Dox ($*P < 0.05$). Cell lysis was delayed when Dox was added in the medium 3 days after infection (open triangle). Mean \pm s.e. of triplicate determination is shown. (b) Crystal-violet-staining image of the cells co-infected with AdCMV-rtTA and AdTRE-E1 with (upper panel) or without (lower panel) Dox. The cells were stained at day 7 after infection.

step, we observed the cell-killing effect at different times of Dox addition in the medium. When Dox was added 3 days after infection, cell lysis was also delayed (c.f. triangle vs. square in Figure 5a). The results suggest that the timing of virus replication can be controlled by the addition of Dox. Without Dox, the infected viruses seemed to be silent, although they were still present in the cells. However, we deduce that the addition of Dox activates the system and reactivates the viruses from sleep. The cell-killing effect was also confirmed by crystal violet staining as shown in Figure 5b.

Tetracycline-inducible gene activation *in vivo*

Finally, we analyzed whether the system works or not *in vivo*. The WI38 cells, human fibroblast-derived cell line, were used in *in vivo* experiments instead of HeLa or A549 cells to avoid the effect of cancer cell metastasis into the organs. The WI38 cells co-infected with AdCMV-rtTA, AdTRE-E1 and AdCMV-luci were transplanted into the peritoneal space in the nude mice. After administration of tetracycline orally, significant higher luciferase activities were shown in the liver, spleen and especially in the mesenterium compared with each organ in the control mice fed with the water without tetracycline as shown in Figure 6. However, no significant difference was seen in the kidney and lung. These data suggest that tetracycline activated the tetracycline-inducible promoter transcriptionally *in vivo*, and the viruses replicated in the presence of E1 protein significantly. These viruses destroyed the WI38 cells and were released into the peritoneal space, then infected in mesenterium and transferred to the liver and spleen through the blood flow from mesenteric vein. On the basis of these data, the tetracycline-inducible system seems to function *in vivo*.

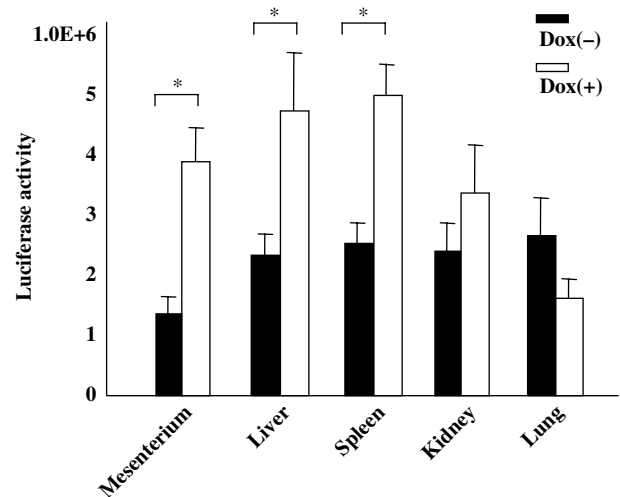


Figure 6 WI38 cells co-infected with AdCMV-rtTA, AdTRE-E1 and AdCMV-luci were transplanted into the peritoneal space in the nude mice. The mice were fed with drinking water with or without Dox for 3 days. Then, the luciferase activity was measured in the liver, spleen, kidney, lung and mesenterium. The luciferase activity in the liver, spleen and mesenterium in the mice fed with Dox (open bar) was significantly higher than in the mice without Dox (closed bar) ($*P < 0.05$), whereas, there was no significant difference in luciferase activity in the kidney and lung. Mean \pm s.e. of triplicate determination is shown.

Discussion

CRADs, which can overcome the insufficient transduction of solid tumor masses by replication-deficient adenovirus, have gained widespread interest in cancer gene therapy. Animal experiments and phase I and II clinical trials

have shown the efficiency and good tolerance of CRAds in cancer gene therapy.^{29–33} However, the risks caused by CRAds should not be ignored. When injected into the body, CRAds induce viremia followed by a strong immune response and extensive cellular injury and death,^{34–36} especially when higher doses are used.^{37,38} Furthermore, it is impossible to control the replication of most currently constructed CRAds externally after injecting them into the body. The pharmacological inducible system is a promising approach to overcome this problem. It has been shown that the E1A gene expression under the control of the mouse mammary tumor virus promoter can be induced by adding dexamethasone or using the rapamycin dimerization system, thus enabling the regulation of CRAd replication.^{39,40} However, in these strategies, the inducers suppress the immune responses, producing potential side effects.

In this study, in an attempt to achieve external control of CRAd replication at an arbitrary timing, we constructed a new rtTA-CRAd system, in which TRE was inserted to the upstream of the E1 gene to control its expression. With AdCMV-rtTA co-infection, CRAd replication could be controlled temporally by adding or removing Dox. The cell-killing assay showed much more obvious cytopathic effect in the cells co-infected with Dox than in those without Dox. The cell proliferation assay also confirmed this result. From an oncolysis standpoint, *in vitro* experiments on the production of progeny released into the supernatant are the most relevant studies for clarifying CRAd effects. In this experiment, we showed that progeny production was 16-fold higher in the rtTA-CRAd system with Dox than in that without Dox. Also, the tetracycline-inducible system was suggested to function *in vivo* as well as *in vitro*. The administration of Dox orally activated the system *in vivo*, and a significant gene expression was observed. Dox is widely used as an antibiotic for the treatment of infectious diseases and also confirmed to be safe clinically.

The synthesis of rtTA can be induced by different tissue-specific promoters. Earlier reports showed that rtTA synthesis enhanced by different tissue-specific promoters increased the selectivity of the expression of targeted genes in the indicated tissue.^{41,42} Conceivably, rtTA under the control of tumor-specific promoters can increase CRAd selectivity in tumor tissue. Thus, using the rtTA-CRAd system, we could control CRAd replication not only temporally but also spatially. In comparison with the one-virus system, another advantage of this system is that it may offer additional safety by reducing the risk of dissemination of replication-competent adenoviruses by requiring the presence of both vectors in a cell to achieve replication competence. Furthermore, various genetic modifications made to replication-deficient adenoviruses, such as adenoviruses with serotype 5 shaft and serotype 3 knob (Ad5/3), and inserting RGD or other peptides into the HI loop, can also be applied to CRAd to overcome coxsackie-adenovirus receptor deficiency in tumor cells. In addition, Dox has already been approved as a drug for

clinical use and it is an orally bioavailable drug with excellent pharmacokinetic properties.⁴³

The main limitation of this system is the presence of intrinsic background activity in the absence of Dox. Both the luciferase activity and cell proliferation assays showed this residual affinity. The substantial high background activity is probably due to the leakiness of the TRE promoter or enhancer function of the terminal repeat sequence of the adenoviral vector. However, a new generation of rtTAs more sensitive to Dox and with low background activity has been developed, thus providing an opportunity to overcome this potential limitation.⁴⁴ Using a tetracycline-controlled transcriptional silencer is another approach to lessen basal level affinity.⁴⁵ Fechner *et al.*⁴⁶ developed an rtTA-rTS-Ad5E1A system and showed low background activity and external control of CRAd replication *in vitro* and *in vivo*. Our *in vitro* and *in vivo* experiments supported their result that the tetracycline-inducible system can be used externally to control CRAd replication.

In summary, using the tetracycline-inducible system, we constructed a novel rtTA-CRAd system and showed its usefulness both *in vitro* and *in vivo* for the external control of CRAd replication and in enhancing safety of CRAds against the immune system in cancer gene therapy.

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