Development of Tetracycline-regulated Adenovirus Expression Vector System

Kyung-Hwa Son[1], Seung-Hoon Lee[1], Jong-Sik Kim[1], Jung-Joo Choi[1], Je-Ho Lee[2]

Recombinant adenovirus vector systems with strong promoters have been used to achieve high level production of recombinant protein. However, this overexpression system cause some problems such as disturbance of cell physiology and increment of cellular toxicity. Here, we showed a tetracycline-regulated adenovirus expression vector system. Our results showed that the expression level of transgene(p-53) was high and easily regulated by tetracycline. In addition, the maximal gene expression level of the tetracycline-controlled gene expression system was higher than that of the wild type CMV promoter system. Therefore, tetracycline-regulated adenoviral vector system could be applicable for regulatory high-level expression of toxic gene. Also, this system will be useful for functional studies and gene therapy.

Keywords: tetracycline-regulated adenovirus, expression vector

INTRODUCTION

The adenovirus gene expression system is useful for basic studies on the analysis of protein function in a wide variety of cells that are difficult to transduce foreign gene by conventional transfection method. However, it has been very difficult to generate recombinant adenoviruses containing cytotoxic gene products under the control of constitutively active promoters (Shockett *et al.*, 1996; Bernard *et al.*, 1998). Such foreign gene products are too toxic for the host cells to propagate the recombinant adenovirus. To overcome this technical difficulty it is desirable to use inducible promoter. Here, we described the construction of the tetracycline-regulated adenovirus vector system. Tetracycline-regulated expression system is widely used for inducible expression in mammalian cells (Gossen *et al.*, 1994; Gossen and Bujard, 1992). Tetracycline-resistance operon of the Tn10 transposon is negatively regulated by tetracycline-repressor(TetR) in *E.coli*. TetR blocks transcription by binding to the operator sequences (tetO) in the absence of tetracycline (Gossen *et al.*, 1995).

[1] Center for Clinical Research, Samsung Biomedical Research Institute, Samsung Medical Center, 50 Ilwon-Dong, Kangnam-Ku, Seoul 135-710, Korea
[2] Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul 135-710, Korea
Correspondence: Je-Ho Lee, Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul 135-710, Korea, Tel: 82-2-3410-3510, Fax: 82-2-3410-0044, E-mail : jeholee@samsung.co.kr

The tetracycline-regulatory expression system basically consists of the two elements (TetR and tetO). These two elements- the TetR protein and tetO regulatory sequence-provided the basis for the tetracycline-regulatory system. The TetR protein fused to the C-terminal domain of the herpes simple virus VP16 as transcription activator, which is called tetracycline tansactivator (tTA) (Gossen *et al.*, 1994). In Tet-Off system, the tTA binds to the seven repetitive tetracycline operator sequences (tetO) and drives transcription of the Tet promoter which consists of a minimal promoter fused to tetO site in the absence of tetracycline. In the Tet-On system, the "reverse" tet repressor (rtTA) was created by four amino acid changes that reverse the proteins response to tetracycline. In the presence of tetracycline, transgene expression is turned on (Molin *et al.*, 1998) (Fig. 1.). Briefly, in the Tet-off system, gene expression is turned on when tetracycline is removed. In contrast, expression is turned on in the Tet-On system by the addition of tetracycline. Both tetracycline-regulated systems, the expression of cloned genes could be tightly regulated in response to tetracycline. Furthermore, the level of maximal expression in Tet system are quite high and compare favorably with the maximal levels obtainable from strong constitutive mammalian promoter such as CMV (Yin *et al.*, 1996). In the Tet-On and Tet-Off system, our study results show that the tetracycline-regulated adenovirus expression system is easily regulated by tetracycline. Furthermore, this system will make it possible to generate recombinant adenoviruses expressing proteins that are cytotoxic or interfere with adenovirus replication (Yoko and

Hirofumi, 1997).

MATERIALS AND METHODS

Cell lines

293A, a human embryonic kidney cell line, was purchased from Quantum and maintained in minimum essential medium Elge's (EMEM) with 10% fetal bovine serum (FBS) (Giboco-BRL, Gaithersburg, USA). The ovarian cancer cell line, SKOV3, was obtained from the American Type Culture Collection and cultured with Dulbecco's Modified Egle's medium (DMEM) supplemented with 10% fetal bovine serum. HeLa-Tetoff, the stable cell line which expresses the tetracycline transactivator (tTA), was obtained from Dr. Yoon (Yonsei University, Seoul) and cultured with same medium used for SKOV-3 cells.

Construction of vectors and generation of recombinant adenovirus

The pTet-on vector, express the reverse tet-responsive transcriptional activator (rtTA) from the strong immediate early promoter of cytomegalovirus (P_{CMV}) was purchased from ClonTech. The pΔAtet*PmimCMV*-p53, pΔACMV-p53 and pΔAtet*PLp*-p53 plasmids were derived from adenovirus type-5 transfer vector (pΔACMV-PA). All plasmids other than pΔACMV-p53 were controlled under the tet-responsive element (TRE) fused with minimal CMV (*PmimCMV*) or L-plastin (*PLp*) promoter. The pΔACMV-p53 was contained the strong promoter (CMV). These plasmids and

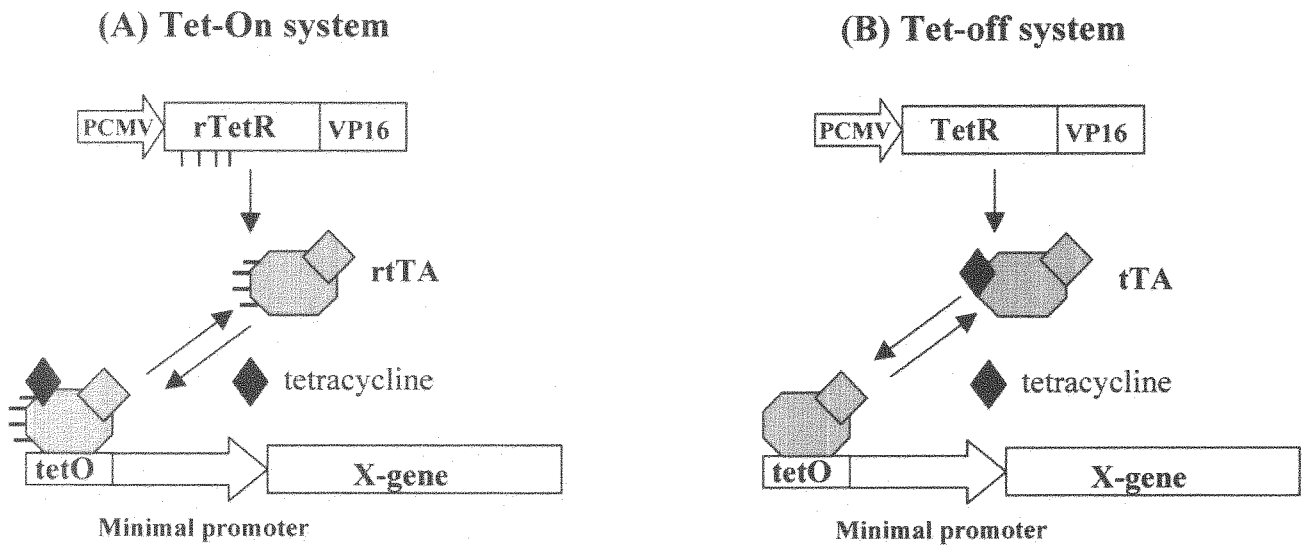

Fig. 1. Schematic diagram of gene regulation in the Tet-On (A) and Tet-Off system (B). The transgene expression is then activated by the addition of the respective inducer. The tetO sequences fused to minimal promoter. (tetO: tetracycline-operator sequences, TetR: tetracycline repressor, VP16: transactivating domain, rTetR: reverse tetracycline repressor, tTA: tetracycline transactivator, rtTA: reverse tetracycline transactivator)

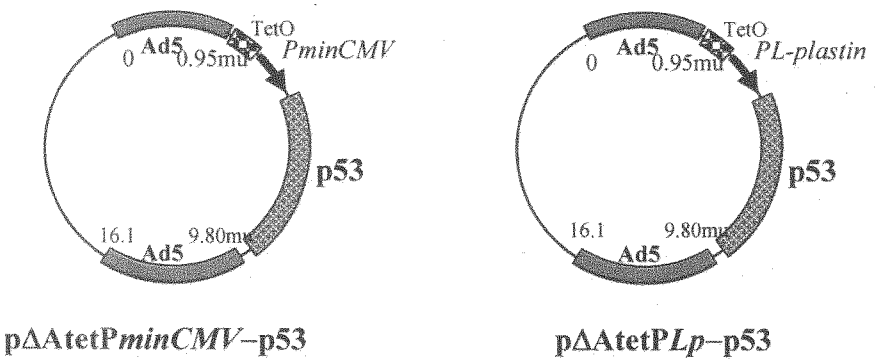

Fig. 2. Constructs of p(AtetPmimCMV-p53 and p(AtetPLp-p53 adnoviral transfer vector. (tet: tetO site, Lp: L-plastin)

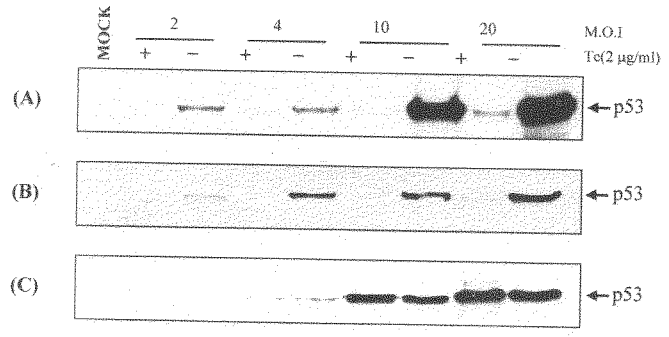

Fig. 3. Regulation of p53 expression in Tet-Off system. HeLa-Tetoff cells were infected with Adv-tetPmimCMV-p53(A), Adv-tetPLp-p53 (B), and Adv-CMV-p53(C) at 2, 4, 8, 10, and 20 pfu per cell. In Tet-off system, the p53 expression is turned on when tetracyclin is removed. (Tc: tetracycline, +: add Tc (2 (/ml), (: not add Tc)

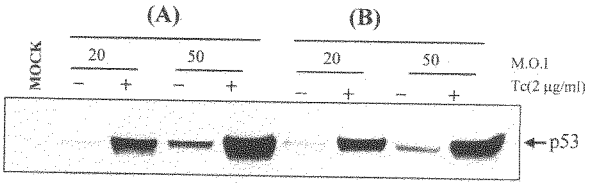

Fig. 4. Regulation of p53 expression in Tet-On system. SKOV cells were transfected with pTet-On vector and were infected with Adv-tetPmimCMV-p53 (A) and Adv-tetPLp-p53 (B) at 20 and 50 pfu per cell. In the presence tetracycline, transgene expression is turned on. (Tc: tetracycline, +: add Tc(2 (/ml), (: not add Tc)

adenovirus genomic DNA plasmid (pJM17) were used for co-transfection into the packing cell line, 293A.The recombinant adenovirus were purified from infected cells and the titers were determined by standard protocols (Massie *et al.*, 1995).

Transfection and western blot analysis

Cells were maintained in 60 mm dishes and infected with adenovirus at the range of 10, 20, and 50 PFU per cell and were transfected the pTet-on vector. After 2 days of infection, total cells were harvested by microcentrfuation at 12,000 rpm for 2 min at 4 °C. The cells were lysed in 200 ul of RIPA buffer (50 mM Tris-Cl, pH8.0, 150 mM NaCl, 1% Nonidet-P 40, 0.5% sodium deoxycholate, 0.1% SDS). 10 ug of proteins were separated in 10% acrylamide gel through SDS-PAGE and transferred to Hybond-ECL nitrocellulose membrane (Amersham, Buckinghamshire, England, UK) and hybridized with anti-p53 antibody. Bands were visualized by enhanced chemoluminescence using the ECL kit(Amersham) according to manufacturer's protocol.

Immunostaining for p53

The p53 gene expression was detected by using monoclonal anti-p53 antibody (Santa cruz Biotechnology, INC.). Immunohistochemical detection was performed using the labeled streptoavidin-biotin method according to the manufacturer's protocol (DAKO, LSAB kit). Incubation with antibodies was carried out at 4 °C for 12 h. DAB at 0.5 mg/ml was used as peroxidase substrate.

RESULTS

The model in Fig. 1. illustrates the regulatory mechanism of gene expression by tetracycline in Tet-on and -off system. To construct the adenovirus vector system which could be effectively regulated gene expression by tetracycline, the tetO fused with a minimal CMV ligated with p53 and inserted into adenovirus type5 transfer vector (pΔACMV-PA)(Fig. 2). And two tetracycline-regulated adenoviral vectors (pΔAtet *PmimCMV*-p53 and pΔAtet*PLp*-p53) were constructed and two recombinant adeno-viruses (Adv-tet*PmimCMV*-p53 and Adv-tet*PLp*-p53) were prepared.

To test the induction of the recombinant adenovirus (Adv-tet*PminCMV*-53, Adv-*tetpLp*-p53, and Adv-*CMV*-p53), HeLa-Tet-off cell line, express the tetracycline transactivator (tTA) was infected at 2, 4, 8, 10, and 20 pfu per cell. Transcription of the p53 gene was blocked by the addition of tetracycline to the culture medium. But the p53 gene expression was induced without tetracycline and was detected at 3 days postinfection by western blot analysis (Fig. 3).

In Tet-Off system, the basal level of p53 expression by Adv-*tetpLp*-P53 was much lower (Fig. 3B) than by Adv-*tetpmin*CMV-p53 (Fig. 3A). But the maximal expression was very high in Adv-*tetpmim*CMV-p53 (Fig. 3A). The p53 gene expression pattern showed that this system was effectively controlled by tetracycline. The increasing amounts of adenovirus resulted in a high expression of p53. However, the basal level of p53 expression did not increase. In addition, the level of maximal p53 gene expression by Adv-tetpminCMV-p53 was much higher than that of the wild type CMV promoter system (Fig. 3).

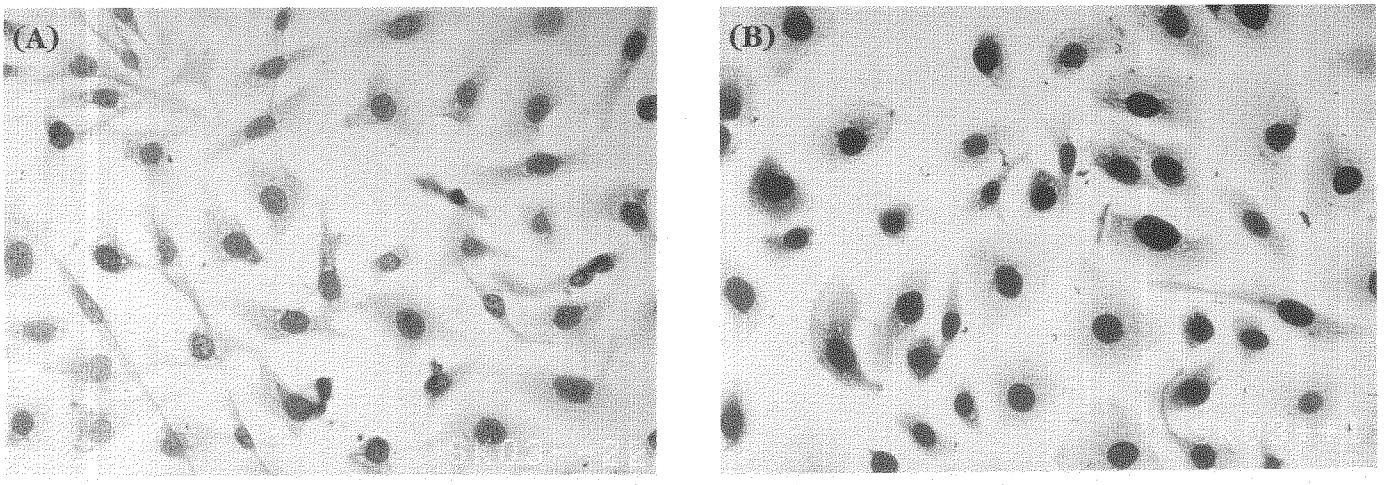

Fig. 5. Immunostaining of the SKOV3 cells treated with Adv-tetPLp-p53 with anti-p53 antibody. (A) Cell line SKOV3 was treated with Adv-tetPLp-p53 at 50 pfu/cell with(+) or without(-) tetracycline.

In Tet-On system, we utilized pTet-On vector for transient rtTA expression. We adopted a transfection-infection strategy to control transgene expression in our system. First, SKOV3, the p53 null cell line, was transfected with pTet-On vector for transient rtTA expression and was infected with recombinant adenoviruses (Adv-TREpminCMV-53 and Adv-TREpLp-p53) at range of 10, 20, and 50 pfu per cell. As shown in Fig. 4., the addition of tetracycline resulted in very high induction of p53 gene in both Adv-TREpminCMV-53 and Adv-TREpLp-p53. Also the basal level of p53 expression was very low in SKOV3.

To test the effect of gene expression controlled by tetracycline we evaluated p-53 expression and the effect in SKOV3 cell line by immunostaining with anti-p53 antibody (Fig. 5). A dramatic increase in p-53 expression was observed in SKOV3 cells treated for one day with Adv-tetPlp-p53 in the presence of tetracycline. Also the cells expressed high p-53 level showed dramatic change of cell shape and developed to death.

In conclusion, we introduced two tetracycline-regulated adenovirus vector systems (Tet-On and Tet-Off). The levels of maximal gene expression in the tetracycline-regulated gene expression system were much higher than that of the wild type CMV promoter system and also gene expression was very effectively regulated by tetracycline.

DISCUSSION

Here, we showed the advantages and efficacy of tetracycline-regulated adenovirus vector system. Adenovirus expression system is already well established as an effective tool for human gene therapy. Therefore, the tetracycline-regulated adenovirus vector may offer unique opportunities for systemic gene therapy of human cancers (Zhang *et al.*, 1997). Most of these reports are based on tetracycline-regulated system using rtTA or tTA -expressing cell lines. But in our studies, an effort of selection of cells expressing the tetracycline-regulated transactivator was eliminated in Tet-On system using pTet-On vector and the maximal gene expression level of the tetracycline-controlled gene expression system was higher than that of the wild type CMV promoter system (Yin *et al.*, 1996; Forster at el., 1998). In our vector systems the gene expression were easily regulated by tetracycline. Therefore, tetracycline-regulated adenoviral vector system will make it possible to express transgenes at significantly low titers, thereby allowing minimization of the toxic side effects associated with the load of adenovirus particles. And also, our vector system will be applicable in a broad range of cell types. Also, tetracycline-regulated adenoviral vector system will be useful for functional studies and could be applicable in gene therapy.

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