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Cytolytic Effects of an Adenoviral Vector Containing L-Plastin Promoter Regulated E1A in Hepatocellular Carcinoma Cells

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Abstract – We have previously reported that 2.4 kb of L-plastin promoter (LP) could regulate the expression of adenoviral vector (AV) exogenous genes in a tumor cell specific manner. In the present study, we tested if the replication competent AdLPE1A vector results in a direct cytotoxic effect in hepatocelluar carcinoma (HCC) cells. *In vitro* cytotoxicity tests were carried out with replication-competent (AdLPE1A) and –incompetent (AdLPCD) LP-driven vectors. AdLPE1A is an AV in which LP was inserted 5' to the E1A and E1B genes. The AdLPCD vector contains LP and the *E. coli* cytosine deaminase (CD) gene in transcription unit. Exposure of cells to AdLPE1A generated a significant cytotoxic effect as compared to the control. Almost 90% of the cell had manifested the characteristic cytopatic effect on day 9 after infection of cells with 10 MOI of AdLPE1A. On the other hand, almost 35% of the cells were left when the cells had been treated with 100 MOI of AdLPCD together with 5-FC on day 9 when compared with the cells which had never been exposed neither 5-FC nor AdLPCD. These results showed that the replication competent AdLPE1A vector could kill the HepG2 cells directly by the oncolytic effect of the virus. The replication competent AV vector carrying viral E1A generated greater cytotoxic effect than the replication incompetent AV, which contains the CD prodrug activation transcription unit without E1A, in HepG2 cells.

Key words □ L-Plastin, Cytosine deaminase, Heptocellular carcinoma, Oncolytic vector, Adenoviral vector

INTRODUCTION

Plastins are a family of human actin-binding proteins that are abundantly expressed in all normal replicating mammalian cells. One isoform, L-plastin, is constitutively expressed at high levels in hematopoietic cell types while T-plastin is constitutively expressed in all non-hematopoietic cells of solid tissues that have replicative potential. L-plastin is, however, constitutively synthesized in many types of malignant human cells of solid tissues indicating that its expression is induced at the time of transformation. Moreover, the activation of L-plastin expression during tumorigenesis appeared to be governed by 5.1 kb of DNA sequence containing promoter and flanking region (Lin *et al.*, 1993a, 1993b; Leavitt, 1994). Based on these reports, we utilized 2.4 kb of L-plastin 5'-flanking region as a transcription unit of AV to achieve transcriptional targeting for cancer. We have shown that it is possible to use of L-plastin promoter (LP) for the regulated expression of an adenoviral exogenous gene in a tumor cell specific manner and spare the normal cells of the body, resulting in the increase of therapeutic index (Chung *et al.*, 1999).

Subsequently, a replication-incompetent adenoviral vector, *i.e.*, AdLPCD, containing a transcription unit of LP and *E. coli* cytosine deaminase (CD) gene was developed (Peng *et al.*, 2001; Chung and Deisseroth; 2004). AdLPCD/5-FC system has been tested in ovary, breast and colon carcinoma so far and shown effective chemosensitization with different extents (Peng *et al.*, 2001; Zhang *et al.*, 2002; Akbulut *et al.*, 2003; Chung 2005). Therefore, AdLPCD viral vector has been suggested to increase 5-FU within tumor cells that are not possible to achieve using systemic administration of 5-FU chemotherapy, thereby increasing therapeutic outcome. However, the most important obstacles to be overcome up for gene therapy

Abbreviations: AV, adenoviral vector; b-gal, b-galactosidase; CD, cytosine deaminase; CMV, cytomegalovirus; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; HCC, hepatocellular carcinoma; LP, L-plastin; MOI, multiplicity of infection; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; X-gal, 5-bromo-4-chloro-3-indoyl-b-D-galactoside.

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protocol including replication incompetent AdLP- vector to work *in vivo* would be insufficient transduction efficiency.

Tissue-specific promoters or enhancers have also been used to regulate the expression of viral genes that are necessary to the replication of AVs, as well as herpes simplex recombinant vectors to increase the number of cancer cells infected by the vector (Rodriguez *et al.*, 1997; Miyatake *et al.*, 1999; Alemany *et al.*, 2000). Zhang *et al.*, (2002) had reported on a conditional replication competent AV in which a 2.4 kb of the LP was placed 5' to the E1A gene of a wild-type adenovirus. The vector generated (AdLPE1A) was directly cytotoxic to the established breast and ovarian cancer cell lines and to primary explant cultures derived from ovarian cancer, but was not cytotoxic to explant cultures of normal mammary epithelial cells. This vector was not cytotoxic to cell lines in which the L-plastin E1A transcription unit was not expressed, whereas the same cell lines were sensitive to the cytotoxic effect of a replication-competent AV in which the cytomegalovirus (CMV) promoter drove E1A expression. Injection of these vectors into nodules, which had been derived from human breast cancer cell, induced regression of these tumors.

Hepatocellular carcinoma (HCC) is the most common internal malignancy in East Asia including Korea and China. Most cancers are diagnosed when they are locally advanced or metastatic and there is no effective treatment available for these patients. Therefore, great expectations are set on gene therapy for the treatment of malignant HCC. In the present study, we tested if the replication competent AdLPE1A vector results in a direct cytotoxic effect in HCC cells.

MATERIALS AND METHODS

Adenoviral vector

AdLPCD (Chung and Deisseroth, 2004) is an E1a-, partial E1b-, partial E3-, linear double stranded AV based on the Ad5 genome. The AdLPCD vector contains the expression cassette consisting of 2.4 kb of the human LP (Chung *et al.*, 1999) and the *E. coli* CD gene in E1 region.

AdLPE1A (Zhang *et al.*, 2002; generous gift of Dr. Deisseroth in Sydney Kimmel Cancer Center, San Diego, CA, USA) is a conditionally replication competent AV in which the 2.4 kb of LP was inserted 5' to the E1A and E1B genes.

The vector for experimentation was prepared by infecting 10 15-cm culture plates of HEK 293 cells and by harvesting the detached cells after 48 hours. The viral particles remained associated with the cells. Cells were collected by centrifugation at

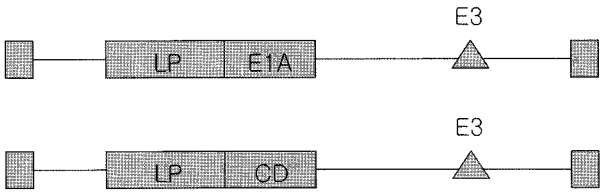

Fig. 1. Demonstration of vectors used in the study. In AdLPE1A vector, the 2.4 kb of L-plastin promoter was inserted 5' to the E1A and E1B genes. AdLPCD contains the CD gene under the control of LP.

400 g for 5 minutes at 4°C. All viral preparations were purified by CsCl density centrifugation, dialyzed, and stored in dialysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM MgCl2) at -70°C prior to use. Titers of the viral stocks were determined by plaque assay using 293 cells by standard methods (Graham and Prevec, 1991). The organization of AdLPE1A is shown in Figure 1.

Cell culture

The cells (HEK 293 and HepG2 cells) were maintained in IMEM supplemented with 10 % fetal bovine serum (FBS) and 0.05% MEME-amino acid.

Cytopatic effect assays

Cells were passaged 24 hours before infection with AV at the indicated MOI. Cells were further maintained in culture for various times. The half of media were removed and replaced with complete fresh media on every 3 days during cell culturing. Photomicrographs were taken on days 3, 6, and 9 after infection.

Chemosensitization assays

Cells were plated in triplicate monolayer a day before the infection. Cells were infected with 100 MOI of AdLPCD in medium containing 2% FBS for 90 min, followed by the addition of 5-FC at 100 μM. Controls included 5-FC (100 μM) alone, AdLPCD alone, or no treatment. Cells were further maintained in culture for various times. The half of media were removed and replaced with complete fresh media containing 100 μM 5-FC on every 3 days during cell culturing. Photomicrographs were taken on days 3 and 9 after treatments.

RESULTS AND DISCUSSION

Cytotoxicity tests in HCC cells following exposure to viral vectors

Conditional replication competent AV could be a strategy for

suppressing uncontrolled cell growth. Since E1A, one of the early adenovirus gene products, is involved in S-phase entry of the infected cells and in adenovirus replication, a tumor-or a tissue- specific promoter integrated upstream to the E1A gene would induce specific killing of target cells.

In this study, *in vitro* cytotoxicity tests were carried out with replication-competent (AdLPE1A) and -incompetent (AdLPCD) L-plastin-driven vectors. The HepG2 human HCC cell line, which has been shown in our laboratory to support the expression of exogenous genes regulated by the LP, was used. The AV transduction of HepG2 cells has been tested using AdLPLacZ, which is a replication-incompetent AV with LacZ reporter gene under the control of LP. As demonstrated by X-gal staining analysis of β-galactosidase activity, AdLPLacZ was able to transduce HepG2 and to express LacZ gene. RT-PCR analyses also have shown that the endogenous *L-plastin* is transcribed in HepG2 cells (paper submitted to the journal).

To test if the replication of the AdLPE1A results in a direct cytotoxic effect, the vector was analyzed for its ability to generate the rounding up and detachment of established human cancer cells. The HepG2 cells were infected with AdLPE1A vector at different MOIs. Cytotoxic effects were compared under the microscope and photos were taken on day 3, 6 and 9 after initiation of treatment. As shown in Fig. 2, exposure of HepG2 cells to the replication competent AdLPE1A vector generated a significant cytotoxic effect as compared to the control. Almost 90 % of the cell had manifested the characteristic cytopatic effect, *i.e.*, rounding up and lifting off of the cell monolayer, on day 9 after infection of cells with 10 MOI of AdLPE1A vector. Infection of higher than 10 MOI of vector did not show extra cytotoxicity, in other words, the vector-dose dependent cytotoxic effects. The maximum predicted cytotoxic effect of the replication competent AdLPE1A vector was seen at an MOI of 10.

In contrast, the weak cytotoxic effect of the AdLPCD replication-incompetent vector was detected (Fig. 3). After exposure of cells to AdLPCD viral vector for 90 min at MOI of 100, 5-FC was introduced at the concentration of 100 μM. Cytotoxic effects were compared under the microscope and photos were taken on day 3 and 9 after initiation of AdLPCD/5-FC treatment. The least cells were left when the cells had been treated with AdLPCD together with 5-FC. However, almost 35 % of the cells were left when the cells had been treated with AdLPCD together with 5-FC on day 9, when compared with the cells which had never been exposed neither 5-FC nor AdLPCD.

These results showed that the replication competent AdLPE1A vector could kill the HepG2 cells directly by the oncolytic effect of the virus. The cytotoxic effect of the replication competent AV vector carrying viral E1A was greater than the replication incompetent AV, which contains the CD prodrug

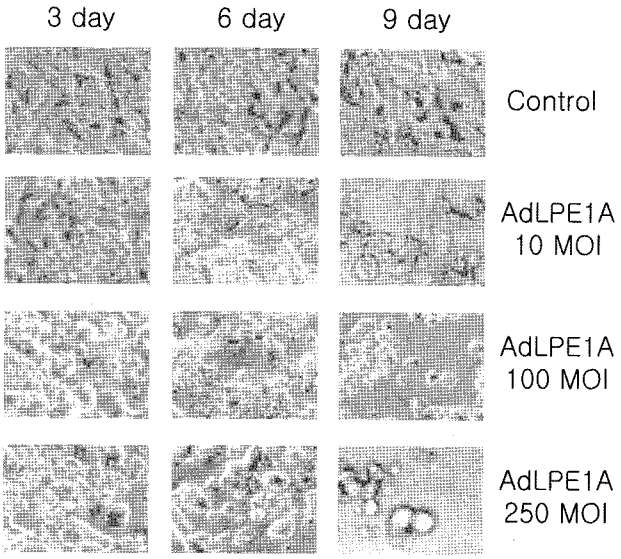

Fig. 2. Study of the sensitivity of HCC cells to the cytotoxic effect of the AdLPE1A vector. The HepG2 cells were exposed to the AdLPE1A vector at an MOI of 10, 100 and 250. Cytotoxic effects were compared under the microscope and photos were taken at day 3, 6 and 9 after initiation of vector treatment.

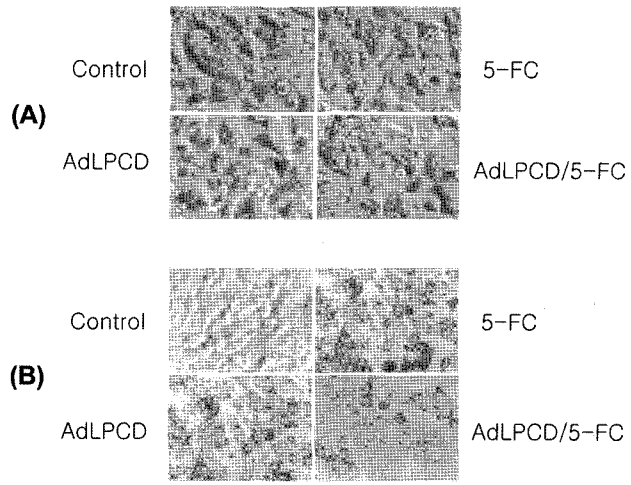

Fig. 3. After exposure of cells to AdLPCD viral vector for 90 min at MOI of 100, 5-FC was introduced at the concentration of 100 μM. (A), (B): Cytotoxic effects were compared under the microscope and photos were taken at day 3 (A) and 9 (B) after initiation of AdLPCD/5-FC treatment.

activation transcription unit without E1A, in HepG2 HCC cells.

A few experiments of AVs carrying the tumor-specific L-plastin driven genes have been reported. In particular, Akbulut *et al.*, (2002) reported that the AdLPE1A was cytotoxic *in vitro* to the MDA-MB-468 established human breast cancer cell lines, destroying the entire monolayer at an MOI of 10, but was not toxic to normal human mammary epithelial cells at the same MOI. The AdLPE1A also caused lifting up and rounding up of all of the cells in explant cultures of ovarian cancer cells obtained from surgical specimens when the vector was added at an MOI of 10. This pattern of cytoxicity suggests that the AdLPE1A vector is selectively toxic to cancer cells but not to normal cells.

It seems that the AdLPE1A vector is much more toxic to breast and ovarian cancer cells than HCC cells, since infection of 10 MOI of AdLPE1A was insufficient to kill entire of HepG2 cells.

These data suggest that the AdLPE1A vector may ultimately be of value for the development of therapeutic vectors for the treatment of solid tumor malignancies including HCC. However, the suppressive effect of the vectors *in vivo* animal study of breast cancer xenograft was not durable (Zhang *et al.*, 2002). Therefore, we are currently under the study with vector (Akbulut *et al.*, 2004) in which L-plastin promoter is driving the expression of E1A gene, which is essential viral replication, and CD gene for the use of treatment of HCC.

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