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# Selective Inhibition of Hepatoma Cells Using Diphtheria Toxin A under the Control of the Promoter/Enhancer Region of the Human α-Fetoprotein Gene

Michito Kunitomi,[1] Eiji Takayama,[1] Satoshi Suzuki,[1] Tatsuji Yasuda,[2] Ken Tsutsui,[2] Kazuhiro Nagaike,[3] Sadayuki Hiroi[4] and Takushi Tadakuma[1, 5]

*[1]Departments of Parasitology and Immunology and [4]Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513, [2]Institute of Cellular and Molecular Biology, Okayama University School of Medicine, 2-5-1 Shikada-cho, Okayama 700-8558 and [3]the Research Center, Mitsubishi Chemical Co., 1000 Kamoshida-cho, Yokohama 227-0033*

**We constructed a plasmid containing human α-fetoprotein (AFP) promoter/enhancer to direct the cell type-specific expression of diphtheria toxin fragment A (DTA), designated as pAF-DTA, to AFP-producing hepatocellular carcinoma cells. The transfection was carried out with cationic liposomes (DMRIE-C) and the expression of the *DTA* gene was confirmed by a northern blot analysis. When pAF-DTA was transfected, the growth of AFP-positive HuH-7 cells was inhibited, whereas growth inhibition was not observed in AFP-negative MKN45 cells. In this experiment, the secretion of AFP was similarly suppressed, but the secretion of carcinoembryonic antigen from MKN45 was not altered. pAF-DTA could also exert its growth inhibitory effect on PLC, a cell line with a low level of AFP. However, no inhibitory effect of pAF-DTA was observed on the proliferation of primary hepatocyte cells. Furthermore, transfection experiments in which HuH-7 and splenic stromal cells were co-cultured revealed the growth inhibition by pAF-DTA to be selective in HuH-7 cells. Finally, the growth of HuH-7 transplanted on BALB/c *nu/nu* mice was inhibited by the direct injection of pAF-DTA/liposome complex into a tumor mass. These results suggest that use of pAF-DTA may be potentially useful as a novel approach for the selective treatment of tumor cells producing AFP even at low levels, without affecting other types of cells.**

Key words: Gene therapy — α-Fetoprotein promoter — Hepatocellular carcinoma — Diphtheria toxin — Tumor-specific expression

Most hepatocellular carcinoma (HCC) cells are characterized by an elevated α-fetoprotein (AFP) level.[1)] AFP is normally expressed in the fetal liver and yolk sac. It is transcriptionally silent in the adult liver but is reactivated in HCC. The promoter and enhancer of *AFP* gene have already been well characterized,[2, 3)] and the use of the AFP transcriptional control sequence provides an ideal tool to achieve HCC-specific gene expression.

The combination of a suicide gene and AFP promoter/enhancer has been recently introduced in the treatment of HCC. In such trials, one well-characterized system is the introduction of herpes simplex virus thymidine kinase (HSV-tk) followed by treatment with the antiviral drug ganciclovir (GCV).[4–6)] One of the limitations of this system is that a rather ample expression of the gene is required for effective treatment. Furthermore, there remains a possibility that the treatment of tumor cells will also injure surrounding normal cells.[7)]

In this study, we introduced the diphtheria toxin fragment A (*DTA*) gene. DTA inactivates elongation factor 2, thus causing an inhibition of protein synthesis.[8)] Once inside the cell, DTA is extremely toxic, and a single molecule is reported to be sufficient to kill a cell.[9)] In addition, even if DTA is released from dying cells, it can not enter other cells in the absence of a B fragment. Cell suicide by the expression of DTA has been demonstrated in cell cultures and transgenic mice,[10, 11)] and the selective expression of DTA by linking the *DTA* gene with tissue-specific transcriptional elements has been reported to be applicable to cancer therapy.[12)] We therefore constructed a plasmid containing the *DTA* gene connected to a 5.1 kb 5′ flanking region of the *AFP* gene (pAF-DTA), and demonstrated that pAF-DTA could exert its cytotoxic effect selectively in AFP-positive HCC regardless of the quantity of AFP produced, without injuring surrounding cells. The advantages and disadvantages of using pAF-DTA for gene therapy of HCC are also discussed.

## MATERIALS AND METHODS

**Cell culture** Two human AFP-positive HCC lines, HuH-7[13)] and PLC/PRF/5(PLC),[14)] and the AFP-negative gastric cancer cell line MKN45[15)] were maintained in culture

[5] To whom correspondence should be addressed.
E-mail: tadakuma@ndmc.ac.jp

flasks in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (Rehatuin Lot No. MB96403, Intergen Co., Purchase, NY). HuH-7 cells are high producers of AFP, whereas PLC cells produce a low level of AFP. The lacZ-positive cell line, GSL, is a stable transformant of the *lacZ* gene established from a splenic stromal cell line which originated from G-CSF transgenic mice,[16] and was kindly supplied by Dr. T. Yamada (Keio University, Tokyo). Human primary hepatocytes were obtained from the Applied Cell Biology Research Institute (Kirkland, WA), and maintained in attachment factor-coated dishes (CS-4ZO-210, Cell Systems Corp., Kirkland, WA) in a CSC serum-free medium (CS-4ZO-800, Cell Systems Corp.).

**Plasmid and plasmid construction** pAF-CAT[3] was kindly supplied by Dr. T. Tamaoki (University of Calgary, Calgary, Canada). It contains a chloramphenicol acetyltransferase (CAT) sequence downstream of 5.1 kilobase pairs of the human *AFP* gene 5′-flanking sequence, containing full enhancer and promoter regions. pLTR-DT contains a gene for the diphtheria toxin A chain under the control of the long terminal repeat (LTR) of bovine leukemia virus as described previously.[17] pAF-DTA plasmid was constructed by replacing the CAT segment at the *Bam*HI site with the DTA fragment obtained from pLTR-DT. Plasmid pAF-DTAR is a control plasmid with reverse insert orientation.

***In vitro* transfection with pAF-DTA** Transfection was carried out with cationic liposomes. On the basis of preliminary experiments, DMRIE-C (GIBCO BRL Life Technologies, Tokyo) was selected and used throughout this study. To observe the inhibitory effect on the cell growth of HCC, the cells were seeded into 6-well plates at a density of $3\times10^4$ cells/well in 2 ml of complete medium 24 h before transfection. A solution of 500 $\mu$l of OPTI-MEM (GIBCO) containing 3 $\mu$g of appropriate DNA and a solution of 500 $\mu$l of OPTI-MEM containing 10 $\mu$l of DMRIE-C were mixed and incubated at room temperature according to the manufacturer's protocol. The cells were washed twice with serum-free OPTI-MEM, and 1 ml of DNA/liposome complex solution was overlaid on the cells. After 5 h of incubation, 1 ml of complete medium containing 20% fetal bovine serum was added to each well. The determination of optimal conditions for transfection was carried out with the lacZ expression vector (*SR$\alpha$-lacZ*) gene in preliminary experiments, and 25–30% of HuH-7 cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) under the conditions described here after 48 h of cultivation. On days 1, 3, 5, and 7, the cells were harvested from each well and the number of viable cells was counted. The results were expressed as the mean cell number of 3 wells. In addition, the AFP concentration in the pooled culture supernatant was determined with a commercially available ELISA kit (Kokusaishiyaku Co., Ltd., Kobe) and the release of carcinoembryonic antigen (CEA) was assessed by using radioimmunoassay (RIA), by SRL Co. (Tokyo).

For transfection to human primary hepatocytes, $5\times10^4$ cells/well were seeded the day prior to transfection. A solution of 500 $\mu$l of OPTI-MEM containing 7.5 $\mu$l of pAF-DTA or pAF-DTAR and a solution of OPTI-MEM with 7.5 $\mu$l of DMRIE-C were mixed, overlaid on the cells, and incubated with the cells for 24 h. After incubation, the solution was replaced and the cells were maintained in the CSC serum-free medium. In the transfection experiment with a mixed culture of HuH-7 and GSL, $1.5\times10^4$ cells of each were mixed and distributed into each well of a 6-well plate 24 h before transfection. At various times after transfection, the number of recovered cells was determined after staining with X-gal, and X-gal-positive cells were counted as GSL, whereas X-gal-negative cells were counted as HuH-7 cells.

**Northern blot analysis** Total cellular RNA was extracted from HuH-7 and MKN45 cells with guanidine thiocyanate followed by centrifugation in cesium chloride solutions.[18] DNA transcripts were detected using a 0.8 kb *DTA* gene and a 1.6 kb *CAT* gene, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA was used as a control.

**Therapeutic effect of pAF-DTA *in vivo*** Female BALB/c *nu/nu* mice were irradiated with 600 rad 10 days before, and were inoculated s.c. into the flank with $1\times10^7$ HuH-7 cells in 50 $\mu$l. Seventeen days after inoculation, mice carrying an estimated tumor volume of 150–200 $mm^3$ (calculated as $1/2\times\text{length}\times\text{width}^2$)[19] were picked up and randomly divided into three groups. DNA/liposome complex (3 $\mu$g of pAF-DTA or pAF-DTAR plus 3 $\mu$l of DMRIE-C in 50 $\mu$l of OPTI-MEM) or phosphate-buffered saline (PBS) was injected directly into the tumor 3 times at 4-day intervals. Tumor sizes were monitored up to 32 days after the first injection of DNA/liposome complex. The animals were killed and the therapeutic effect was evaluated on the basis of tumor weight and histological findings of excised tumors. The AFP concentration in the serum of excised mice was also determined.

## RESULTS

***DTA* gene expression under the control of promoter/enhancer of human *AFP* gene** To confirm the expression and specificity of the *DTA* gene under the control of the *AFP* gene promoter/enhancer, we performed northern blot analysis with DTA and CAT probes. DTA transcripts were clearly detected in HuH-7 cells 6 h after initiation of transfection, and the expression was gradually reduced (Fig. 1A). In contrast, expression of the *DTA* gene was not observed in MKN45 cells transfected with pAF-DTA or in HuH-7 cells transfected with pAF-CAT. Moreover, *CAT*

gene expression was detectable in HuH-7 cells transfected with pAF-CAT, but not in HuH-7 cells with pAF-DTA or in MKN45 cells with pAF-CAT (Fig. 1B). These results clearly indicate that the genes downstream of the AFP promoter/enhancer were expressed selectively in the AFP-producing cells.

**Selective inhibition of cell growth in AFP-positive cells by pAF-DTA** AFP-positive HuH-7 cells and AFP-negative MKN45 cells were transfected either with pAF-DTA or with pAF-DTAR. As shown in Fig. 2, the cell growth of HuH-7 cells with pAF-DTA was significantly suppressed as compared to the growth of HuH-7 cells with pAF-DTAR. In contrast, no marked difference was observed in cell growth between MKN45 cells with pAF-DTA and those with pAF-DTAR. This differential effect was not due to reduced efficiency of transfection into MKN45 cells. In two independent experiments, the transfection efficiencies estimated from X-gal staining were 33% in MKN45 cells as compared to 25% in HuH-7 cells and 28% in MKN45 cells to 23% in HuH-7 cells, respectively.

In addition, the effects of pAF-DTA on the production of AFP from HuH-7 cells and CEA from MKN45 cells were investigated (Fig. 3). AFP released in the supernatant was reduced in HuH-7 cells with pAF-DTA, while the production of CEA from MKN45 was not affected by transfection with either pAF-DTA or pAF-DTAR.

We next investigated whether pAF-DTA could exert its inhibitory effect even on cells producing low levels of AFP. PLC is a low producer of AFP, and in our preliminary experiments, $10^6$ HuH-7 cells in a 2 ml culture medium released 57.8 ng/ml of AFP after overnight cultivation, whereas the release of AFP was 3.8 ng/ml from PLC cells. As shown in Fig. 4, the growth of the PLC cells was also significantly suppressed when the cells were transfected with pAF-DTA as compared to those with pAF-DTAR.

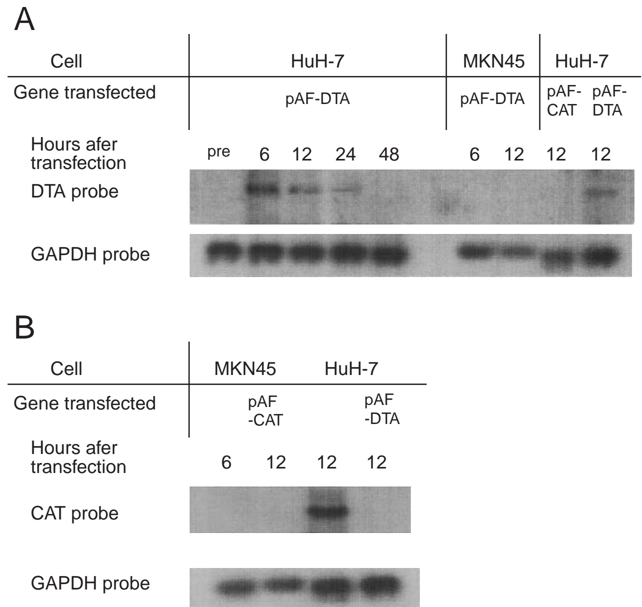


Fig. 1. Northern blot analysis of *DTA* gene expression. pAF-DTA or pAF-CAT was transfected with cationic liposomes (DMRIE-C) into AFP-positive HuH-7 cells or AFP-negative MKN45 cells. The total RNA was extracted from the cells at an appropriate time after initiating the transfection, and 10 $\mu$g of RNA were applied to each lane. The DNA transcripts were detected using either 0.8 kb *DTA* gene (Fig. 1A) or 1.6 kb *CAT* gene (Fig. 1B). GAPDH DNA was used as a control.

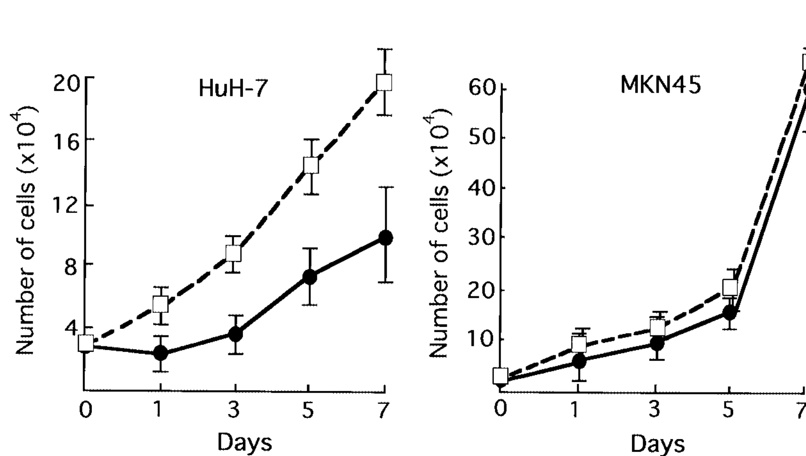


Fig. 2. Selective inhibition of cell growth in HuH-7 cells by transfection with pAF-DTA. HuH-7 or MKN45 cells were seeded into 6-well plates at a density of $3\times10^4$ cells/well the day before transfection. pAF-DTA (●) or pAF-DTAR (□) was transfected as described in "Materials and Methods." On days 1, 3, 5, and 7 of cultivation, the cells were harvested and the results were expressed as the mean cell number±SD of 3 wells.

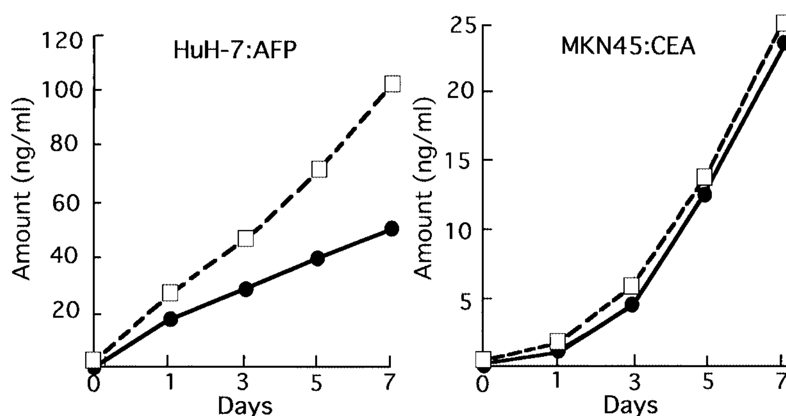


Fig. 3. Effect of pAF-DTA on the release of AFP or CEA. The culture supernatants were collected from pAF-DTA-transfected (●) or pAF-DTAR-transfected (□), HuH-7 or MKN45 cells in Fig. 2. The AFP and CEA concentrations were determined with ELISA and RIA assays, respectively from the pooled supernatants of 3 wells.

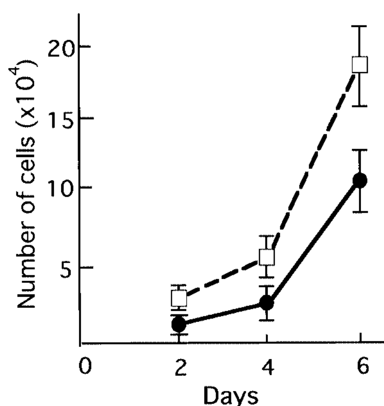


Fig. 4. Inhibition of cell growth in PLC cells, a low producer of AFP. PLC cells were seeded into 6-well plates at a density of $3\times10^4$ cells/well the day before transfection. pAF-DTA (●) or pAF-DTAR (□) was transfected to PLC cells as described in "Materials and Methods." On days 2, 4, and 6 of cultivation, the cells were harvested and the results were expressed as the mean cell number±SD of 3 wells.

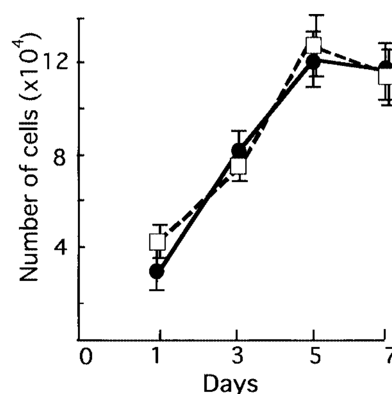


Fig. 5. Effect of pAF-DTA on the proliferation of primary hepatocytes. Primary hepatocytes were commercially obtained, and were seeded into 6-well plates at a density of $5\times10^4$ cells/well the day before transfection. The cells were transfected with pAF-DTA (●) or pAF-DTAR (□)/liposome complex for 24 h, and afterward maintained in the CSC serum-free medium. On days 1, 3, 5, and 7 of cultivation, the cells were harvested, and the results were expressed as the mean number of cells±SD from 5 wells.

**Effect of pAF-DTA on primary hepatocytes** One of the problems of gene therapy using the 5′ flanking region of the *AFP* gene is that it might also be harmful to intact hepatocytes, especially proliferating hepatocytes during both reparative and regenerative growth. We investigated the inhibitory effect of pAF-DTA on the proliferation of primary hepatocytes. However, we observed no significant difference in cell growth between the cells with pAF-DTA and those with pAF-DTAR (Fig. 5). This phenomenon was not due to the inability of pAF-DTA/liposome complex to transfect into hepatocytes. In our preliminary experiments with *lacZ* gene transfection, 15–20% of hepatocytes were stained with X-gal under this experimental condition.

**Selective activity of pAF-DTA in the mixed cell culture** The inhibitory effect of pAF-DTA was tested in a mixed culture of HuH-7 cells and splenic stromal cells, GSL. Since GSL cells are X-gal-positive (>95%), the total number of recovered cells and the ratio of X-gal-positive and negative cells were determined. The results in Fig. 6 indicated that the suppressive effect of pAF-DTA was limited to AFP-positive cells and did not affect the surrounding GSL cells.

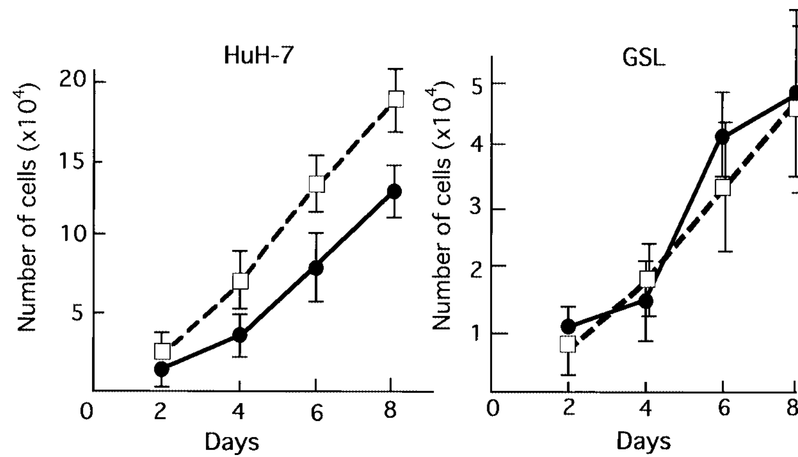


Fig. 6. Effect of pAF-DTA on the cell growth in a mixed culture of HuH-7 and GSL cells. AFP-positive HuH-7 cells and lacZ-positive GSL cells were mixed and seeded in 6-well plates. The mixed cells were transfected with pAF-DTA (●) or pAF-DTAR (□), and the number of recovered HuH-7 cells and GSL cells was determined after staining with X-gal. The results were expressed as the mean cell number±SD of 3 wells.

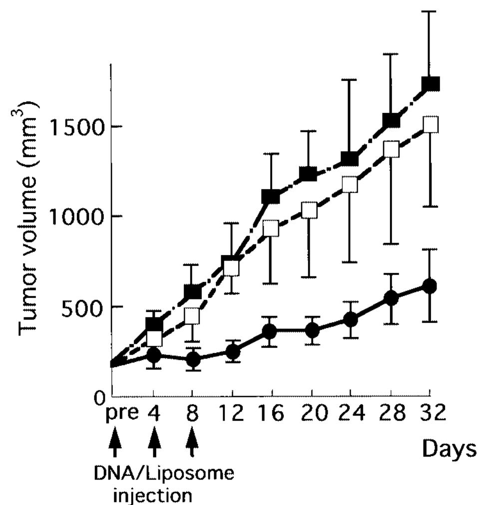


Fig. 7. Inhibition of tumor growth by pAF-DTA *in vivo*. On the 10th day after receiving X-irradiation (600 rad), female BALB/c *nu/nu* mice were inoculated s.c. with $1\times10^7$ HuH-7 cells. When the estimated tumor volume reached 150–200 $mm^3$, the mice were randomly divided into three groups. PBS (■), pAF-DTA/liposome complex (●), or pAF-DTAR/liposome complex (□) was injected directly into the tumor mass three times at 4-day intervals. The growth of the tumor was then assessed up to 32 days and the results were expressed as the mean volume±SD of 5 mice.

**Therapeutic effect of pAF-DTA *in vivo*** To test the cytotoxic effect of pAF-DTA on AFP-producing tumors *in vivo*, HuH-7 cells were inoculated s.c. into BALB/c *nu/nu* mice. When the estimated tumor volume reached 150–200 $mm^3$, the mice were divided into 3 groups, and DNA/liposome complex or PBS was directly injected into the HCC tumor three times at 4-day intervals. The extent of tumor growth was observed up to 32 days after the initiation of treatment. As shown in Fig. 7, growth inhibition was observed in the mice with pAF-DTA as compared to the mice with pAF-DTAR or PBS. A comparison of the excised tumor weight showed that the average tumor weight from pAF-DTA-treated mice was one-fourth of that from pAF-DTAR-treated mice (Table I). Furthermore, the AFP concentrations in the mice treated with pAF-DTA decreased to one-third of those from pAF-DTAR-treated mice. Histological examinations of tumor specimens revealed massive cell death in the central area of the tumor in pAF-DTA-treated mice (unpublished observation).

Table I. Excised Tumor Weight and Serum AFP Level in Mice Treated with pAF-DTA[a)]

| Group | Treatment | Tumor weight (g) | Serum level of AFP (ng/ml) |
|---|---|---|---|
| 1 | pAF-DTA | 0.62±0.21[b)] | 565±97.4[b)] |
| 2 | pAF-DTAR | 2.30±0.66 | 1626±240.2 |
| 3 | PBS | 2.07±0.67 | 1621±265.0 |

*a)* The mice described in Fig. 7 were killed 32 days after the initiation of treatment, and the excised tumor weight and serum level of AFP were determined. The data were expressed as the mean±SD of 5 mice.
*b)* Significantly different from groups 2 or 3 by Student's *t* test ($P<0.01$).

## DISCUSSION

In this study, we demonstrated that pAF-DTA could inhibit cell growth of AFP-positive HCC, HuH-7 and PLC, *in vitro* and *in vivo*. In contrast, pAF-DTA did not exert any significant effect on AFP-negative MKN45 or primary hepatocytes.

To determine the cell type-specific expression of DTA, we used the whole 5.1 kb upstream region of AFP. Besides the AFP-specific promoter, this region contains two enhancers and two silencers (strong and weak ones).[20, 21] In previous experiments, the portion containing the silencers was generally omitted mainly due to the limited package capacity of either adenovirus or retrovirus vectors.[4, 22] It was interesting to note that the silencer activity was low in both fetal liver and AFP-producing HCC, but was highly activated in adult liver.[21] It is therefore essential to maintain the silencer activity in order to protect intact liver cells which might be injured by transfection of pAF-DTA. We herein demonstrated that pAF-DTA did not exert an inhibitory effect on the proliferation of primary hepatocytes. In this experiment, the release of AFP was not detectable by ELISA or RIA (assessed by SRL Co.) assay (unpublished data). Over the years, it has been assumed that the proliferation of hepatocytes during reparative and regenerative growth is associated with retro-differentiation,[23] which is revealed by AFP expression. A recent paper,[24] however, reported that hepatocyte proliferation in the adult liver is not associated with dedifferentiation. If this is the case, it will be highly beneficial for pAF-DTA, from the viewpoint of clinical application.

As a suicide gene, *HSV-tk* or *Escherichia coli* cytosine deaminase has been used.[4–6, 22] The expression of the genes in the transfected cells transforms nontoxic GCV or 5-fluorocytosine into toxic GCV triphosphate or 5-fluorouracil. One of the limitations of these suicide gene systems is that the cytotoxic effect is dependent on both the number of genes transfected and on the transcriptional activity of the promoter/enhancer of the suicide gene.[4, 5, 25] If the suicide gene is introduced by an adenovirus vector, a relatively large number of adenovirus particles is necessary for infection. To overcome this limitation, some devices including the introduction of Cre-loxP mediated recombination have been considered.[26] In contrast, DTA is a strong inhibitor of protein synthesis and it is reported that even a single molecule can kill a cell.[9] We herein demonstrated that the growth of PLC, a low producer of AFP, was also significantly inhibited by pAF-DTA. Another advantage of using DTA is that the target cells do not have to divide actively. Furthermore, if there is an unintended toxicity arising from the basal expression in non-targeted cells, it is possible to replace DTA with a less potent, attenuated version.[27]

Another difference between HSV-tk/GCV or cytosine deaminase/5-fluorocytosine and DTA is the presence of a bystander effect.[4, 28] Cells expressing the *HSV-tk* gene can induce cell death in neighboring cells which do not express HSV-tk. This is called the bystander effect and is caused by the gap-junction-mediated transfer of cytotoxic molecules to bystander cells.[29] As a result, 10% of transfected cells was enough to exert a cytotoxic effect on all the cells maintained *in vitro*.[6] This bystander effect is beneficial when the suicide genes are directly injected into the tumor itself.[30] However, it injures the surrounding cells. In contrast, DTA is retained within the cells, and even if it is released from dying cells, it can not enter other intact cells in the absence of B chain. In the mixed culture of HuH-7 cells and splenic stromal cells, pAF-DTA selectively inhibited the growth of HuH-7 cells, but the growth of stromal cells was not influenced. This characteristic is considered to be highly advantageous for targeted gene therapy. In the future, it should be possible to administer suicide genes systematically instead of by direct administration into the tumor mass.

In addition we expect our suicide gene therapy to be a useful prophylactic treatment. Clinically it is sometimes observed that AFP level in the serum is high, but tumor nodules are not detectable. In such cases, pAF-DTA will selectively eliminate AFP-producing cells which might be potentially oncogenic.

Finally, as a carrier of the suicide gene, we employed cationic liposomes. It is sufficient to express suicide genes transiently in the cells, and adenovirus has been widely used as a vector.[31] Adenovirus vectors can be produced in high titers and they efficiently transfer genes to replicating and non-replicating cells. However, adenovirus vectors can evoke nonspecific inflammation and can also easily induce strong anti-vector immunity. Therefore, repeated systematic administration is not practical. Moreover, a recombinant adenovirus has a size limitation of the inserted gene of up to 7 to 8 kb.[31]

Liposomes have many advantages as a gene transfer vector. They can deliver large pieces of DNA, as shown in our experiment (the size of our plasmid was 9.87 kb). They also evoke fewer inflammations or immune responses. As liposomal preparations, various cationic liposomes are commercially available and they are used as plasmid-liposome complexes.[32] In this experiment too, such complexes were used for transfection. For *in vivo* administration, however, it is better for the plasmids to be entrapped in the liposomes in order to escape enzymatic attack or other problems in the circulation. These liposomes can be surface-modified so that they may be delivered selectively to the target cells by conjugating monoclonal antibodies or other devices.[33] For ideal gene therapy, two critical points of selectivity still need to be resolved, i.e., the selective delivery of the plasmid vehicle

to the target cells and the selective expression of the desired gene in the targeted cells. This paper and others have demonstrated that cell-type-specific expression is feasible by using the promoter and enhancer regions of the genes which are selectively expressed in the desired cells. We are now focusing our efforts on developing liposomes equipped with targeting and fusion activities to hepatocytes.

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