Gene Therapy for Carcinoembryonic Antigen-producing Human Lung Cancer Cells by Cell Type-specific Expression of Herpes Simplex Virus Thymidine Kinase Gene

Tadashi Osaki, Yoshiro Tanio, Isao Tachibana, Shigeto Hosoe, Toru Kumagai, Ichiro Kawase, Shinzo Oikawa, and Tadamitsu Kishimoto

Department of Medicine III, Osaka University Medical School, 2-2, Yamaooka, Suita, Osaka 565 [T. O., Y. T., I. T., S. H., T. Ku, I. K., T. Ki.], and Santory Institute for Biomedical Research, 1-1-1, Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618 [S. O.], Japan

Abstract

A carcinoembryonic antigen (CEA)-producing human lung cancer cell line (A549), a nonproducing human lung cancer cell line (CADO-LC9), and a human uterine cervical cancer (HeLa) were transfected with the herpes simplex virus thymidine kinase (HSV-TK) gene regulated by 445 nucleotides upstream from the translational start of CEA gene. Fifty % growth inhibitory concentration of ganciclovir (GCV) was 0.57 μM for HSV-TK-transfected A549; relative sensitivity to GCV was more than 1000 times higher compared to the 50% growth inhibitory concentration of the parental cell line. Both CADO-LC9 and HeLa transfected with HSV-TK were still resistant to GCV. There was no difference in either morphology or doubling time between HSV-TK-transfected and parental clones. Further, injections (i.p.) of GCV resulted in significant regression of HSV-TK-transfected A549 tumors in nude mice. These data show the possibility of gene therapy using the cell type-specific promoter of CEA gene against CEA-producing adenocarcinoma of the lung.

Introduction

Lung cancer is one of the most refractory solid tumors because inoperable cases are up to 60% and the 5-year survival is only 13% (1). In particular, adenocarcinomas, which comprise about one-half of the total lung cancer cases, are mostly chemo-radioresistant.

Recently, gene therapy consisting of HSV-TK3 gene transfer followed by GCV administration has been applied for the experimental tumors (2—5). One advantage of this therapy is that it requires a second treatment, GCV injection, which enables us to decide the most desirable time to start therapy. GCV is proved to be safe in many clinical cases. GCV is phosphorylated in cells transfected with HSV-TK gene and incorporated into DNA as guanosine analogue, stopping DNA synthesis and inducing cell death (2, 3). Finally, a phenomenon, the so-called “bystader effect,” may be observed. When a part of the tumor cell population has been transfected to express HSV-TK gene, neighboring nontransduced tumor cells could be also damaged after GCV administration (5). However, one of the important problems of the HSV-TK and GCV system may be how to enhance the specificity to tumor cells (6, 7). Although it is known that retroviral vector is an useful tool for gene delivery both in vitro and in vivo, it is impossible to transfer genes exclusively into tumor cells without affecting surrounding normal cells, other than the central nervous system (4), because the discrimination of the gene transfer depends on cell proliferation. To overcome this limitation, we have investigated the usefulness of a cell type-specific promoter to express HSV-TK gene selectively in a human lung adenocarcinoma cell line.

CEA has been widely used as a tumor marker of various carcinomas since the report of Gold and Freedman in 1965 (8). Lung cancer, about one-half of the total cases are serologically positive for CEA, although a high concentration (20 ng/ml <) of CEA is detected mostly in adenocarcinomas (9). In 1987, cDNA of CEA was cloned in several laboratories (10—12); since then, cDNAs of closely related family members, such as NCAs, have been cloned. On the basis of the sequence similarity, the CEA gene family is now divided into two main subgroups; the CEA subgroup contains genes encoding CEA itself, NCA, and BGP1 (13). The expression patterns of CEA-related genes were investigated with Northern blot analysis. CEA and NCA were often coexpressed in various adenocarcinomas and adenosmas. Kim et al. (14) reported that normal lung tissue expressed NCA RNA in 7 of 8 cases, but they failed to detect CEA and BGP1 RNAs. On the other hand, Schrewe et al. (15) cloned the complete gene for CEA and analyzed its putative promoter region. In this region of CEA and NCA genes, homology was found, but a high sequence divergence was also detected in the 240 nucleotides upstream of the translational start. Schrewe et al. (15) demonstrated that the putative CEA promoter region which contained about 400 nucleotides upstream from the translational start exhibited an apparently higher promoter activity in SW403 (producing both CEA and NCA) than in nonproducing HeLa. Thus, cell type-specific expression of the CEA gene might be possibly conveyed by this promoter region.

We amplified the CEA promoter region by PCR and ligated it with the promoterless HSV-TK gene. This new chimera gene was transfected into a CEA-producing adenocarcinoma cell line, A549. The A549-expressing HSV-TK was shown to be highly sensitive to GCV; i.p. injections of GCV resulted in significant regress of the A549 grown s.c. in nude mice. These results suggest that gene therapy for the refractory adenocarcinoma of the lung might be possible under the appropriate cell type-specific promoter.

Materials and Methods

Cell Lines. The human adenocarcinoma cell line of the lung, A549, was obtained from American Type Culture Collection (Rockville, MD). CADO-LC9 was established and supplied by T. Sakuma (Osaka University, Osaka, Japan). RERF-LC-MS, RERF-LC-OK, VMRC-LCD, and ABC-i were provided by Japan Cancer Research Resources (Tokyo, Japan). The human uterine cervical cancer cell line, HeLa, was a gift from Dr. M. Fujiwara (Biomedical Research Center, Osaka University, Osaka, Japan). The human adenocarcinoma cell line of the lung, OAD3, and the human small cell lung cancer cell line, OS2R, were established in our laboratory (16). All cell lines except for OS2R were established in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (General Scientific Laboratories, Los Angeles, CA), 100 units/ml penicillin (Meiji Seika, Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika), and 2 mM L-glutamine (Flow Laboratories, North Lyde, Australia), which was designated CM. OS2R was maintained in CM supplemented with 10 nm hydrocortisone, 5 μg/ml.
insulin, 10 μg/ml transferrin, 10 nm 17β-estradiol, and 30 nm sodium selenite, which was supplemented with 5% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (16). These cell lines were free of mycoplasma contamination as tested on Bacto PPLO Agar (Difco Laboratories, Detroit, MI).

**CEA Protein in Cell Homogenate.** Cells were washed and suspended in phosphate-buffered saline and homogenized with sonicator at 50 W for 2 min. CEA protein was analyzed in cell homogenates by solid phase radioimmunoassay with anti-human CEA polyclonal antibody; protein concentration was assayed by Lowry’s method (Specific Reference Laboratories, Tokyo, Japan).

**Plasmid Construction.** The cis-acting sequences which convey cell type-specific expression of the CEA gene are contained in the region upstream of between −424 and −2 base pairs from the translational start as described by Schrewe et al. (15). A fragment which contains this putative CEA promoter region was amplified by PCR with a pair of primers, which had extraneous nucleotides including BglII at sites at the 5’ end (sense primer, 5’-CCAGATCTCATCCTCCCATCCAGAGCC; antisense primer, 5’-TACCCAG- GAGACCCCAOCCAGT). The amplified fragment was cut with NcoI. After generation of blunt ends with mung bean nuclease, the fragment was recut with BglII and inserted into the upstream of pSV000CAT (Nippon Gene, Tokyo, Japan), resulting in the construct pCEACAT. To create CEA promoter HSV-TK chimer gene (pCEATK-S), the BglII/PvuII fragment of pTK4, about 373 A DNA sequencer, Applied Biosystems, Foster City, CA).

**Results**

**Expression of CEA Gene in Human Lung Cancer Cell Lines.** Northern blot analysis was performed with specific cDNA probes (Fig. 1). Three bands were detected with the CEA probe. The 4.2- and 3.5-kilobase bands are mRNAs of CEA and shown in A549, LoVo, RERF-LC-MS, and VMRC-LCD. The 2.9-kilobase band is a mRNA of NCA (20) and is detected only in A549 and LoVo, which was also confirmed by a NCA-specific probe. RERF-LC-MS and VMRC-LCD showed weak expression of CEA mRNA. The colon cancer cell line LoVo revealed the strongest expression of CEA mRNA. On the other hand, CEA was not expressed in CADO-LC9, RERF-LC-OK, ABC-1, OS2R, or HeLa. CEA-related proteins in cell homogenate were also analyzed. Among lung cancer cell lines, A549 showed the highest concentration (138 ng/mg protein). RERF-LC-MS and VMRC-LCD expressed a relatively low concentration (10.4 and 31.7 nm/mg protein, respectively). No detectable CEA-related proteins were present in
In vivo Effect of GCV on Cell Lines Transfected with pCEATK. To examine whether these HSV-TK-transfected clones are also sensitive to GCV in vivo, 2 × 10^6 tumor cells were inoculated s.c. at the flank of nude mice and treated with GCV (Fig. 3). A549/TK-S34, a clone of the HSV-TK-transfected A549 cells, grew as well as did A549 parental cells in nude mice (group 1). The growth of the cloned cells was significantly suppressed by repeated i.p. injections of GCV (group 2). Neither A549/TK-AS10, a clone of A549 cells given the anti-sense TK gene, nor A549 parental cell line was sensitive to the GCV treatment (groups 3 and 4). Similar experiments were performed using the HeLa cell line. HeLa cells transfected with pCEATK gene grew as did the parental HeLa cells in mice given GCV treatment (data not shown). Thus pCEATK works specifically in the CEA-producing tumors and kills such cells following GCV treatment both in vitro and in vivo. No apparent body weight loss was observed in mice treated with GCV. All mice but one in group 2 recurred after cessation of GCV treatment. To make clear the mechanism of recurrence, three of the recurrent tumors were excised and tested on GCV sensitivity in vitro, and their genomic DNA was extracted to see whether integrated pCEATK was lost or not. All 3 tumors still contained HSV-TK gene and showed GCV sensitivity identical to the original A549/TK-S34 clone (data not shown). The same dose of GCV was then repeatedly reijected into reserved mice with tumor recurrence. However, the relapsed tumors did not regress again (data not shown). These results indicate recurrent tumor cells were resistant to GCV in spite of retaining functional pCEATK. A549 cells were mixed with A549/TK-S34 cells at a ratio of 1:1, and then inoculated s.c. into nude mice (Fig. 3, group 5). The parental cells still produced growing tumors even when inoculated into mice as a mixture with A549/TK-S34 cells, and there was no significant difference in the growth rate between A549 tumor and tumors produced by the mixture, although a slight retardation of growth was observed in another experiment (data not shown).

Discussion

CEA has been one of the most promising tumor markers for tumor-specific targeting therapy until it was shown to be expressed in normal colonic mucosa (13). However, CEA is a good monitoring
marker for the conventional anticancer therapy, since some tumors produce much higher concentration of CEA than normal tissues (13). The precise control mechanisms of CEA expression in tumor cells have not been elucidated, although hypomethylation in the upstream element of CEA gene was shown to be correlated with high expression of CEA (21). On the other hand, it was reported that cis-acting sequences conveying cell type-specific expression might be within the putative promoter region of CEA by comparing a CEA-producing colon cancer cell line with the nonproducing HeLa (15). We decided to use this putative CEA promoter as the regulator of HSV-TK expression in order to devise tumor-specific gene therapy against CEA-producing lung adenocarcinomas, and showed that the plasmid pCEATK rendered CEA-expressing lung cancer cells susceptible to GCV both in vitro and in vivo.

There was a significant difference in the CAT activity of the CEA promoter fragment between A549 and HeLa, but not between A549 and CADO-LC9. The CAT activity of pCEACAT in other CEA-producing cancer cell lines, LoVo and OAD3 showed 17% and 21% of pSV2CAT, respectively. One of the reasons for such low activity may be that the fragment is too short to contain tissue-specific enhancer or silencer sequence. More specific expression could be achieved by using the tissue-specific enhancer or silencer sequences that may exist in introns or far upstream of 5'-region of CEA. Nevertheless the CEA promoter ligated to HSV-TK gene resulted in favorable cell type-specific expression in the GCV sensitivity tests. There is a discrepancy between data from the CAT assay and that from the GCV sensitivity tests in CADO-LC9 and HeLa. Although CADO-LC9 appears to have stronger CEA promoter activity than HeLa, the GCV sensitivity of HeLa is greater than that of CADO-LC9. However, 58.3 μM of GCV in HeLa/TK-S may not be acceptable with regard to considering in vivo efficacy, because Paul et al. reported that the nontoxic concentration of GCV was 15–18 μM (22). Therefore, this discrepancy seems to be meaningless in vivo. One of the reasons for the discrepancy may come from the fact that the CAT assay shows transient expression of CAT gene while the GCV sensitivity reveals permanent expression of HSV-TK gene in our present study. Another reason for it seems to be that CADO-LC9 may be naturally more resistant to GCV than HeLa. CEA expression in human adenocarcinoma cell lines was investigated by Northern blot analysis. Although the PstI fragment of CEA cDNA used as the probe in this study cross-reacts to NCA and BGPI, it was possible to discriminate CEA mRNA from other two mRNAs by the transcript size. We also distinguish NCA mRNA from other two mRNAs by a NCA-specific probe. We showed that half of the lung adenocarcinoma cell lines, including A549 and RERF LC-MS, expressed CEA mRNA. These data were consistent with the report by Kim (14). However, Hasegawa et al. (23) reported that neither A549 nor RERF LC-MS expressed CEA mRNA, although they detected CEA protein with Western blot and fluorescence-activated cell sorter analysis (23). This difference may be based in part on the fact that RNA undergoes degradation more rapidly than protein.

In the present study, tumor reoccurred after cessation of GCV treatment, although HSV-TK gene was still integrated in the genomic DNA of tumor cells and expressed. It is most likely that small number of tumor cells were out of the cell cycle during GCV treatment and then reentered the cell cycle after cessation of treatment. However, the tumor burden was too large to be eliminated by the initial dose of GCV. We tried dose escalation up to 75 mg/kg of GCV and observed the growth suppression of some recurrent tumors (data not shown). Tumor recurrence after GCV treatment has been already reported by other investigators, even after 1 month of continuous GCV treatment (24). Moreover, according to the information about toxicity of GCV supplied by Nippon Syntax, 8 of 50 mice died during the treatment with 45 mg/kg of GCV once daily for 30 days. Thus, dose escalation or combination treatment with other drugs which act in different ways (rather than longer treatment periods) should be considered.

We have shown the new approach to CEA-producing lung cancer cells which are usually refractory to conventional chemotherapy. Although some normal tissues (such as gastrointestinal and colorectal tissues) other than the lung express CEA protein, this method may be applicable to local therapy using bronchoscopy or thoracentesis.

References

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