ELSEVIER

Cancer Letters 96 (1995) 105–110

CANCER LETTERS

Gene therapy of hepatoma: bystander effects and non-apoptotic cell death induced by thymidine kinase and ganciclovir

Yoshiyasu Kaneko*, Ayumi Tsukamoto

First Department of Medicine, Faculty of Medicine, University of Tokyo 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

Received 26 June 1995; accepted 25 July 1995

Abstract

A retroviral vector carrying herpes simplex virus thymidine kinase gene was constructed, and transfected into the $\psi 2$ packaging cells. The replication-defective retrovirus produced by this cell line ($\psi 2$tkn cells) was transduced into XC rat hepatoma cells, from which a cell line (XCtkn2) highly sensitive to ganciclovir was cloned. Ganciclovir suppressed the growth of XCtkn2 hepatoma and $\psi 2$tkn cells. Both of these HSV-tk-carrying cells treated with ganciclovir showed potent 'bystander effect' on co-culturing with genetically unmodified XC hepatoma cells. In addition, intratumoral injection of XCtkn2 and $\psi 2$tkn cells into the XC hepatomas transplanted in nude mice and subsequent ganciclovir administration suppressed in vivo growth of the hepatomas. Flow cytometry disclosed that the ganciclovir-treatment increased the relative number of XCtkn2 hepatoma and $\psi 2$tkn cells at the G2 phase of the cell cycle. However, the nuclear fragmentation and internucleosomal DNA cleavage were not observed, indicating that the death of XCtkn2 hepatoma and $\psi 2$tkn cells treated with ganciclovir was not apoptotic.

Keywords: Apoptosis; Cell cycle; Flow cytometry; Gene therapy; Hepatocellular carcinoma; Retroviral vector; Thymidine kinase gene

1. Introduction

Continuing advances in molecular biology are likely to allow the development of new cancer treatments and methods of cancer prevention that will redefine cancer therapy [4]. One of the promising approaches in the rapidly growing area of gene therapy is the 'virus-directed enzyme/prodrug therapy' using viral vectors, herpes simplex virus (HSV) thymidine kinase (tk) gene and the anti-viral reagent ganciclovir (GCV) [16]. Transfer of HSV-tk gene into brain tumors has been achieved by direct injection of the tumor with a cell line actively producing a retroviral vector carrying the gene conferring drug sensitivity to the tumor [3,17]. In rats with metastatic liver tumors, the tumor regression is recognized by an intratumoral injection of packaging cells producing HSV-tk gene-expressing recombinant retroviral particles and GCV treatment [1,7]. These suggest that the HSV-tk/GCV system may be promising for gene therapy of various cancers including hepatoma [1,7, 16]. The HSV-tk/GCV system is known to interfere in DNA synthesis. GCV is changed into the mono-

* Corresponding author.

0304-3835/95/$09.50 © 1995 Elsevier Science Ireland Ltd. All rights reserved
SSDI 0304-3835(95)03919-N

phosphate form by HSV-tk, which is further converted to the triphosphate form by cellular kinases. GCV triphosphate is incorporated into DNA molecules and prevents further DNA elongation [7]. However, subsequent processes leading to cell death are not always clear. In the present study, therefore, the effects of GCV and the cells carrying HSV-tk gene on the in vivo and in vitro growth of rat hepatoma were examined with reference to the mechanism of cell killing.

2. Materials and methods

2.1. Construction of a retroviral vector

A retroviral vector carrying the HSV-tk gene was constructed from a plasmid pHSV 106 (Gibco BRL, Gaithersburg, MD) and a Moloney murine leukemia virus-derived vector pZIPneo [2]. In brief, pHSV106 carrying HSV-tk gene was digested with BamHI. HSV-tk gene was separated by agarose gel electrophoresis, and inserted into BamHI site of pZIPneo. The resulting retrovirus vector (pZIPtkn) was mixed with lipofectin (Gibco BRL) and transfected into the $\psi 2$ packaging cell line [13]. The cells carrying the neomycin resistant gene were selected by culturing in DMEM + 5% fetal bovine serum (FBS) containing 500 μg/ml of geneticine (G418). The retrovirus produced by these cells ($\psi 2$tkn cells) was transduced to XC rat hepatoma cells under the presence of 5 μg/ml of polybren. HSV-tk-transduced XC (XCtkn) cells were selected by culturing under the presence of 500 μg/ml of G418. A cell line (XCtkn2) highly sensitive to GCV was cloned from the XCtkn cells by limiting dilution using 96-well culture plates.

2.2. Hepatoma growth in vivo and in vitro

Effects of GCV on the growth of XC, XCtkn, XCtkn2 and $\psi 2$tkn cells were examined by culturing these cells (5×10^5 per 60 mm dish) in DMEM + 5% FBS. The effects of the HSV-tk/GCV system on the in vivo growth of XC hepatoma cells were investigated by the intratumoral injection of XCtkn2 and $\psi 2$tkn cells into the transplanted XC hepatoma and GCV treatment. Briefly, XC hepatoma cells (2×10^7) were transplanted into 6 weeks-old ICR nude mice (Nisseizai, Tokyo). After the tumors were established, XCtkn2 or $\psi 2$tkn cells (1×10^7) were injected four times into the XC hepatomas. GCV (30 mg/kg body weight) was injected intraperitoneally.

2.3. Flow cytometry and DNA fragmentation

The flow cytometric analysis of the DNA contents of GCV-treated cells was carried out as described [9,10]. In brief, XCtkn2 and $\psi 2$tkn cells (5×10^5) were cultured for different durations of time with or without GCV (20 μg/ml), harvested by trypsin-treatment, and stained with propidium iodide (5 μg/ml). These cells were subjected to flow cytometry using the FACScan/cell FIT DNA system (Becton Dickinsson). Nuclei were observed under the fluorescent microscope after the cells were cultured for different periods of time with 20 μg/ml of GCV, fixed and stained with acridine orange (1 μg/ml). To investigate whether cell death was associated with an internucleosomal DNA fragmentation characteristic to apoptosis, DNAs were extracted by the phenol method and subjected to electrophoresis in 2% agarose gels. The DNAs were stained with 1 μg/ml of ethidium bromide and their gel electrophoretic patterns were photographed [9,10]. To investigate whether the apoptosis occurs in XCtkn2 and $\psi 2$tkn cells, they were cultured with mitomycin C (1 μg/ml) in RPMI1640 medium + 5% FBS and their DNAs were subjected to agarose gel electrophoresis.

3. Results

As demonstrated in Fig. 1A, GCV inhibited the growth of XCtkn and $\psi 2$tkn cells but not that of genetically unmodified XC cells. Most of the $\psi 2$tkn cells were killed within 3–4 days of culture with 20 μg/ml of GCV, while XCtkn cells reduced the cell number only by 50% after 6 days of GCV-treatment (Fig. 1A). Since this partial sensitivity of XCtkn cells to GCV was supposed to be due to the presence of low producers of HSV-tk, those cells highly sensitive to GCV were cloned by limiting dilution. Among several cell clones tested, clone 2 (XCtkn2 cells) was most sensitive to GCV (Fig. 1B). Co-cultures of XCtkn2 or $\psi 2$tkn cells with the genetically unmodified XC hepatoma cells were carried out to examine the bystander effect of the HSV-tk/GCV system. Both XCtkn2 and $\psi 2$tkn cells were effective in suppressing the growth of the unmodified XC cells (Fig. 1C). The in vivo effects of HSV-tk-producing cells

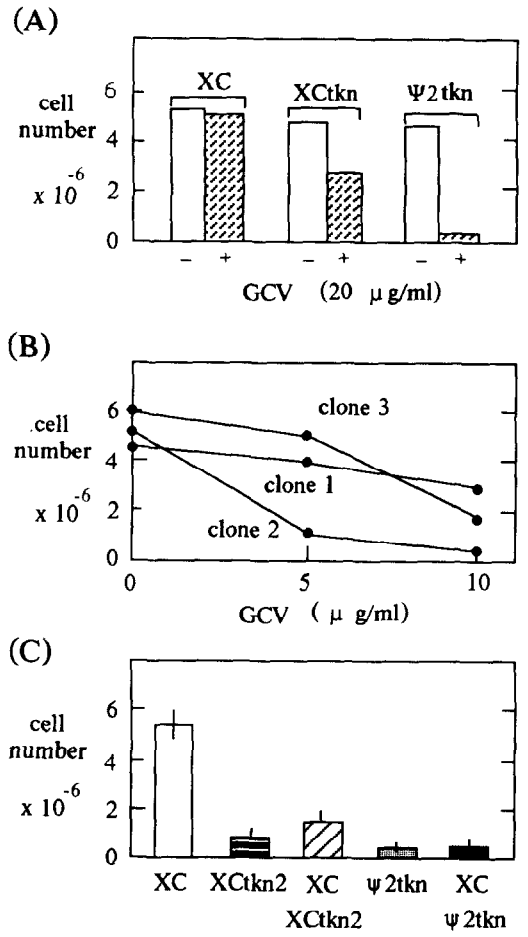

Fig. 1. Effects of the HSV-tk/GCV system on the in vitro growth of XC and XCtkn hepatoma and ψ2tkn cells. (A) XC hepatoma, XCtkn, and ψ2tkn cells (5×10^5 cells/dish) were cultured for 6 days with (+, hatched bars) or without (–, open bars) 20 μg/ml of GCV. (B) Clones of XCtkn cells (5×10^5 cells/dish) obtained by limiting dilution were tested for their sensitivity to GCV by culturing for 6 days under the presence of 0, 5 and 10 μg/ml of GCV. Clone 2 (XCtkn2) was most sensitive to GCV. (C) XC hepatoma (5×10^5 cells/dish), XCtkn2 (5×10^5 cells/dish), and ψ2tkn (5×10^5 cells/dish) cells were cultured independently or co-cultured for 6 days under the presence of 20 μg/ml of GCV.

on the XC hepatomas transplanted into the nude mice were demonstrated in Fig. 2. The transplantation of XC hepatoma cells into the nude mice, intratumoral injections of XCtkn2 and ψ2tkn cells and intraperitoneal administrations of GCV were carried out as demonstrated in Fig. 2A. At the start of the intratumoral injection of tk-producing cells, XC hepatomas were approximately 1 cm in diameter. The inratumoral injection of cells and GCV treatment were repeated as indicated in Fig. 2A. The weight of XC hepatomas at the end of the experiment was 10.1 ± 1.5, 4.3 ± 1.09 and 2.8 ± 1.3 g in control, XCtkn2-injected and ψ2tkn-injected tumors, respectively (Fig. 2B).

To clarify the mechanism of cell killing by the HSV-tk/GCV system, the effects of GCV on the DNA content, nuclear morphology, and DNA fragmentation of XCtkn2 and ψ2tkn cells were examined (Figs. 3–5). The flow cytometry of propidium iodide-stained nuclei disclosed that most of ψ2tkn cells treated for 3 days with GCV (20 μg/ml) were arrested

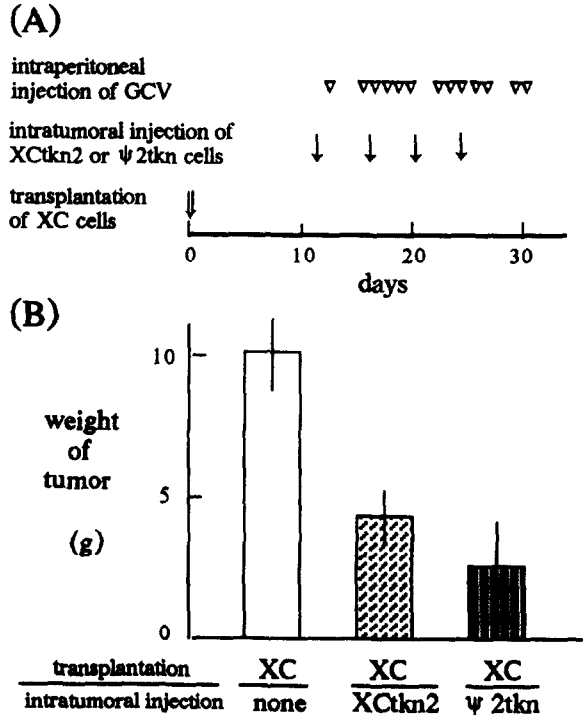

Fig. 2. Effects of intratumoral injection of HSV-tk-producing cells on the in vivo growth of XC rat hepatoma cells. (A) Experimental protocol. XC hepatoma cells (2×10^7) were transplanted into nude mice (day 0). XCtkn2 cells (1×10^7) or ψ2tkn cells (1×10^7) were injected intratumorally at days 12, 16, 20 and 24 (arrows). GCV (30 mg/kg body weight) was administered intraperitoneally as indicated by arrow heads. At the 33rd day from the transplantation of XC cells, mice were killed and the tumors were weighted. (B) The growth of XC hepatoma cells which were transplanted into nude mice and treated with intratumoral injection of XCtkn2 and ψ2tkn cells and intraperitoneal administration of ganciclovir as shown in (A). The results are the mean ± SD ($n = 5$).

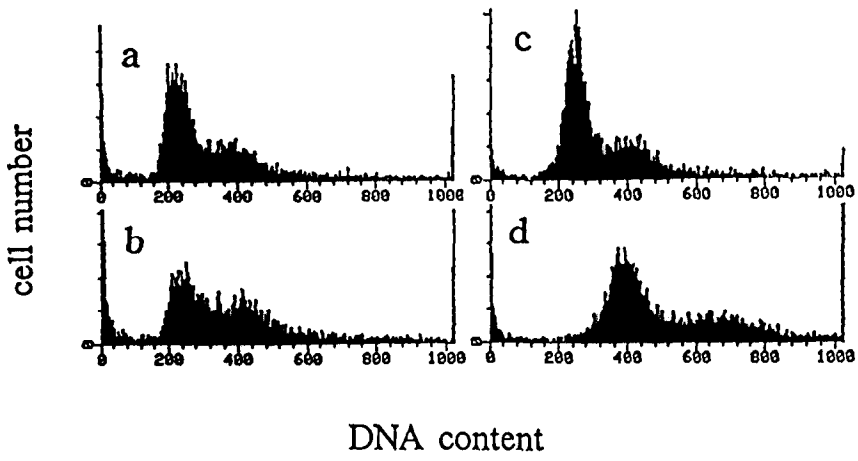

Fig. 3. Flow cytometric analysis of DNA content of XCtkn2 and ψ2tkn cells treated with GCV. XCtkn2 (a,b) and ψ2tkn (c,d) cells (5×10^5 cells/dish) were cultured for 3 days without (a,c) or with (b,d) 20 μg/ml of GCV. The cells were harvested, incubated with propidium iodide and subjected to one-color flow cytometry.

at the G2 phase of cell cycle. In contrast, the GCV-induced G2 arrest in XCtkn2 cells was not so remarkable as in ψ2tkn cells. Nonetheless, the GCV-treatment reduced the relative number of XCtkn2 cells in the G1 phase of the cell cycle and increased that of cells in the G2 phase within 3–4 days (Fig. 3). These GCV effects were recognized in three independent experiments using either XCtkn2 or ψ2tkn cells. Small nuclei with less DNA content, which reflected fragmented ones characteristic to apoptotic cell death, were not recognized in the flow cytometric analysis (Fig. 3). On the other hand, GCV had no remarkable effects on the flow cytometric patterns of the nuclear DNA content of the genetically unmodified XC cells.

The fluorescent microscopic study revealed that the size of the nuclei of cells treated for 2–3 days with GCV was larger than that of control cells (Fig. 4b,e). This was followed by reduction of their nuclear size, but the nuclear fragmentation and chromatin condensation characteristic to apoptosis were not recognized (Fig. 4c,f). Subsequently, the cells became rounded and began to detach from the substratum of the culture dish. Many of these free-floating cells retained the ability to exclude trypan blue dye for at least 1–2 days. Agarose gel electrophoresis of DNAs demonstrates that the DNA ladders characteristic to apoptosis were not recognized in those DNAs from GCV-treated XCtkn2 and ψ2tkn cells in either the monolayer or free-floating state (Fig. 5). In contrast, the DNA ladder was clearly observed in the DNAs from XCtkn2 and ψ2tkn cells

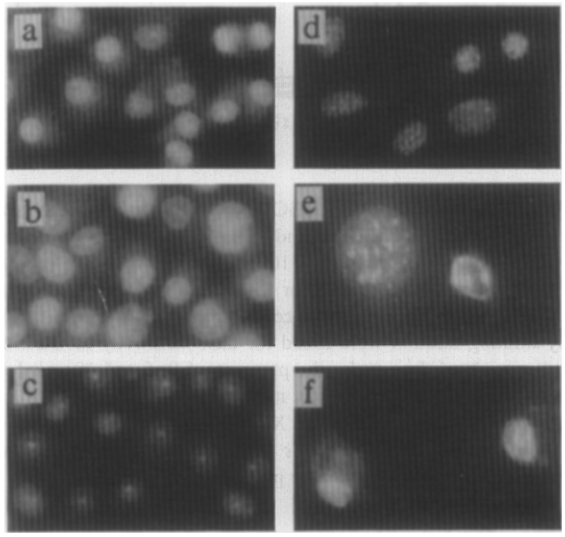

Fig. 4. Effects of GCV on the nuclear morphology of XCtkn2 and ψ2tkn cells. XCtkn2 cells (5×10^5 cells/dish) were cultured for 3 days without GCV (a), or cultured for 3 days (b) or 4 days (c) with 20 μg/ml of GCV. ψ2tkn cells (5×10^5 cells/dish) were cultured for 3 days without GCV (d), or cultured for 3 days (e) or 4 days (f) with 20 μg/ml GCV. Nuclei were stained with acridine orange and photographed under the immunofluorescent microscope (×400).

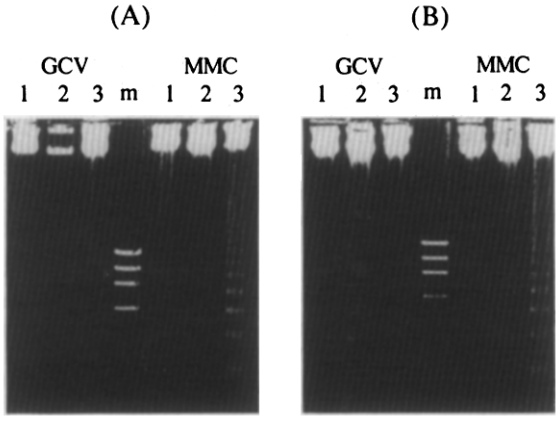

Fig. 5. Gel electrophoresis of DNAs of XCtkn2 and ψ2tkn cells cultured with GCV or mitomycin C (MMC). (A) XCtkn2 cells (5×10^5/dish) were cultured for 3 (lane 1), 4 (lane 2) and 5 (lane 3) days with 20 μg/ml of GCV in DMEM + 5% FBS (left) or with 1 μg/ml of MMC in RPMI1640 + 5% FBS (right). (B) ψ2tkn cells (5×10^5/dish) were cultured for 2 (lane 1), 3 (lane 2), and 4 (lane 3) days with 20 μg/ml of GCV in DMEM + 5% FBS (left) or with 1 μg/ml of MMC in RPMI 1640 + 5% FBS (right). DNAs were extracted by the phenol method and electrophoresed in 2% agarose gels with ϕX174RN DNA/HaeIII digests (lane m) as molecular size markers.

cultured with mitomycin C (1 μg/ml) for 4–5 days in RPMI1640 medium + 5% FBS. This indicated that the GCV-induced DNA damage did not trigger the apoptotic process in XCtkn2 and ψ2tkn cells even though these cells had apoptotic cell death mechanisms (Fig. 5).

4. Discussion

As demonstrated in the present study, GCV inhibited the growth of HSV-tk gene-transduced XC hepatoma and ψ2 cells. In addition, the HSV-tk/GCV system showed a potent bystander effect on the genetically unmodified XC hepatoma cells in vivo and in vitro. The bystander effect of the virus-producing ψ2tkn cells was more remarkable than non-producing XCtkn2 cells, suggesting that in addition to the cell-to-cell transfer of phosphorylated GCV, new transduction of the retrovirus carrying HSV-tk gene into the neighboring XC hepatoma cells was responsible for their potent killing effect [4]. In our present experimental system, however, the retroviral transduction to XC hepatoma cells in vitro was not effective under the absence of polybren. Therefore, the marked inhibitory effect of ψ2tkn cells on hepatoma growth was supposed to be due mainly to the cell-to-cell transfer of phosphorylated GCV and other still unknown mechanisms [1,3,6]. Recent studies in experimental animals suggested that the bystander effect might mediate a tumor eradication by GCV even if a substantial number of tumor cells failed to acquire the HSV-tk gene [15]. The present in vivo experiment shown in Fig. 2 demonstrates that the intratumoral injection of XCtkn2 and ψ2tkn cells into the transplanted XC hepatomas was also effective in inhibiting the growth of the latter tumors (Fig. 2). These suggest that the HSV-tk/GCV system and similar gene therapy protocols may be clinically feasible for patients with an advanced cancer.

The HSV-tk/GCV system changed the flow cytometric patterns of cellular DNA content. Our present study shown in Fig. 3 clearly demonstrates that almost all GCV-treated ψ2tkn cells were arrested at the G2 phase of the cell cycle. The HSV-tk/GCV system terminates DNA elongation by an integration of phosphorylated GCV [15]. The cells with incompletely duplicated DNAs cannot generate signals to trigger the mitosis, and stay at the G2 phase of the cell cycle to complete the DNA duplication [18]. When the DNA damage and abnormality were severe and unrepairable, the cell death process is triggered [12]. On the other hand, the G2 arrest induced by the HSV-tk/GCV system was not so remarkable in the case of XCtkn2 cells (Fig. 3). The reduced G2 arrest is one of the characteristics of cancer cells, which causes an accumulation of carcinogenic mutation. This may be related to the fact that tumor cells activate cyclin B-cdc2 regardless of the state of the DNA and enter mitosis with damaged DNA [5,8].

The GCV-induced death of glioma cells has been reported to be apoptotic because of the appearance of DNA ladders on gel electrophoresis which are characteristic to apoptosis [11]. As shown in the present study, however, the death of XCtkn2 hepatoma and ψ2tkn packaging cells caused by the HSV-tk/GCV system was not associated with the internucleosomal DNA fragmentation, indicating that their death appeared not to be apoptotic. Since the apoptotic death mechanisms existed in both XCtkn2 and ψ2tkn cells, it is supposed that the DNA damage induced by HSV-tk/GCV did not produce signals to trigger apoptosis in these cells. The triggering mechanisms of

non-apoptotic cell death may be different depending on the kinds of cells and also on the in vivo and in vitro experimental systems, and the exact reason for the discrepancy between the past report and the present result is not known [11,19]. Understanding of this type of cell death in addition to apoptosis may be important to improve anticancer therapy [5,12,14]. Further studies are necessary to provide knowledge about the cell death mechanism and the new strategy for gene therapy of hepatoma.

References

[1] Caruso, M., Panis, Y., Gagandeep, S., Houssin, D., Salzmann, J.L. and Klatzmann, D. (1993) Regression of established macroscopic liver metastasis after in situ transduction of a suicide gene. Proc. Natl. Acad. Sci. USA, 90, 7024–7028.

[2] Cepko, C.L., Roberts, B.E. and Mulligan, R.C. (1984) Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell, 37, 1053-1062.

[3] Culver, K.M., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H. and Blaese, R.M. (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of brain tumors. Science, 256, 1550–1552.

[4] Culver, K.W. (1994) Clinical applications of gene therapy for cancer. Clin. Chem., 40, 510–512.

[5] Fisher, D.E. (1994) Apoptosis in cancer therapy: crossing the threshold. Cell, 78, 539–542.

[6] Freeman, S.M., Abbound, C.N., Whartenberg, K.A., Packman, C.H., Koeplin, D.S., Moolten, F.L. and Abraham, G.M. (1993) The 'bystander effect': tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res., 53, 5270–5283.

[7] Huber, B.E., Richards, C.A. and Krenitsky, T.A. (1991) Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. Proc. Natl. Acad. Sci. USA, 88, 8039–8043.

[8] Hunter, T. and Pines, J. (1994) Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell, 79, 573–582.

[9] Kaneko, Y. and Tsukamoto, A. (1994) Apoptosis and p53 expression in human hepatoma cells induced by etoposide, mitomycin C and thapsigargin. Int. Hepatol. Commun., 2, 305–309.

[10] Kaneko, Y. and Tsukamoto, A. (1995) Apoptosis and nuclear levels of p53 protein and proliferating cell nuclear antigen in human hepatoma cells cultured with tumor promoters. Cancer Lett., 91, 11–17.

[11] Kato, K., Yoshida, J., Mizuno, M., Sugita, K. and Emi, N. (1994) Retroviral transfer of herpes simplex thymidine kinase gene into glioma cells causes targeting of ganciclovir cytotoxic effect. Neurol. Med. Chir. Tokyo, 34, 339–344.

[12] Kerr, J.F., Winterford, C.M. and Hamon, B.V. (1994) Apoptosis. Its significance in cancer therapy. Cancer, 73, 2013–2026.

[13] Mann, R., Mulligan, R.C. and Baltimore, D.B. (1983) Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell, 33, 153–159.

[14] Martin, S.J. and Green, D.D. (1994) Apoptosis as a goal of cancer therapy. Curr. Opin. Oncol., 6, 616–621.

[15] Moolten, F.L. (1994) Drug sensitivity ('suicide') genes for selective cancer chemotherapy. Cancer Gene Ther., 1, 279–287.

[16] Moolten, F.L. and Wells, J.M. (1990) Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. J. Natl. Cancer Inst., 82, 297–300.

[17] Oldfield, E.H., Ram, Z., Culver, K.M., Blaese, R.M., DeVroom, H.L. and Anderson, W.F. (1993) Gene therapy for the treatment of brain tumor using intratumoral transduction with thymidine kinase gene and intravenous ganciclovir. Hum. Gene Ther., 4, 39–69.

[18] Williams, G.T. (1991) Programmed cell death: apoptosis and oncogenesis. Cell, 65, 1097–1098.

[19] Vile, R.G., Nelson, J.A., Casleden, S., Chong, H. and Hart, I.R. (1994) Systemic gene therapy of murine melanoma using specific expression of the HSV-tk gene involves an immune component. Cancer Res., 54. 6228–6234.