Mechanisms of Cell Death Induced by Suicide Genes Encoding Purine Nucleoside Phosphorylase and Thymidine Kinase in Human Hepatocellular Carcinoma Cells In Vitro

TIM U. KROHNE,1 SRINIVAS SHANKARA,2 MICHAEL GEISSLER,1 BRUCE L. ROBERTS,3 JACK R. WANDS,3 HUBERT E. BLUM,1 AND LEONHARD MOHR1

For gene therapy of hepatocellular carcinoma (HCC), the Escherichia coli purine nucleoside phosphorylase (PNP)/fludarabine suicide gene system may be more useful than the herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) system as a result of a stronger bystander effect. To analyze the molecular mechanisms involved in PNP/fludarabine-mediated cell death in human HCC cells in comparison with HSV-tk/GCV, we transduced human HCC cells of the cell lines, HepG2 and Hep3B, with PNP or HSV-tk using adenoviral vectors, followed by prodrug incubation. Both systems predominantly induced apoptosis in HepG2 and Hep3B cells. PNP/fludarabine induced strong p53 accumulation and a more rapid onset of apoptosis in p53-positive HepG2 cells as compared with p53-negative Hep3B cells, but efficiency of tumor cell killing was similar in both cell lines. In contrast, HSV-tk/GCV–induced apoptosis was reduced in p53-negative Hep3B cells as compared with p53-positive HepG2 cells. HSV-tk/GCV, but not PNP/fludarabine, caused up-regulation of Fas in p53-positive HepG2 cells and of Fas ligand (FasL) in both HCC cell lines. These results demonstrate cell line–specific differences in response to treatment with PNP/fludarabine and HSV-tk/GCV, respectively, and indicate that PNP/fludarabine may be superior to HSV-tk/GCV for the treatment of human HCC because of its independence from p53 and the Fas/FasL system. (HEPATOLOGY 2001;34:511-518.)

Tumor therapy based on the expression of suicide genes has become an attractive therapeutic strategy for human malignancies such as hepatocellular carcinoma (HCC),1-6 which is a malignant tumor with increasing incidence.7,8 HCC is highly resistant to chemotherapeutic agents and radiotherapy,9 which may be caused by molecular changes occurring during oncogenesis such as overexpression of the multidrug resistance gene10 and loss or mutations of tumor suppressor genes.11,12 Suicide genes induce tumor cell death by the conversion of nontoxic prodrugs into highly toxic metabolites and may also affect nontransduced cells (bystander effect).13 Suicide gene–induced tumor cell death may occur by apoptosis or necrosis.14 Typical features of apoptosis are nuclear condensation, DNA fragmentation, or cleavage of caspase substrates such as poly(ADP-ribose) polymerase (PARP).15 Apoptotic cell death is mediated by a variety of pathways that involve receptor ligand interactions and intracellular signaling cascades leading to the activation of caspases, followed by the activation of DNA fragmentation factor and caspase-activated deoxyribonuclease. By comparison, necrosis may be defined as a mechanism of cell death, in which the classical features of apoptosis are absent.14 Understanding the precise mechanisms involved in tumor cell death induced by a therapeutic approach may be of great importance, because tumor cells may acquire genetic changes during oncogenesis. Such cellular alterations may result in the loss of cell-cycle control or resistance to apoptosis induced by anticancer agents via mutations of the p53 tumor suppressor gene,16,17 or as a result from alterations of the Fas/FasL signal transduction pathway.18-20 Antitumor strategies involving or entirely depending on pathways frequently altered in tumor cells may therefore result in a loss of therapeutic efficiency. To improve the efficiency of suicide genes and to avoid resistance of tumor cells toward suicide gene–mediated cell death, the understanding of the precise mechanisms involved in suicide gene–mediated tumor cell killing is most important.

Herpes simplex virus thymidine kinase (HSV-tk) is the prototype suicide gene. Analysis of tumor cell death induced by the HSV-tk/ganciclovir (GCV) system in various human and murine tumor cell lines has demonstrated apoptosis in HSV-tk–expressing cells, as well as in nontransduced bystander cells.21,22 In some cell lines, signal transduction pathways such as the CD95/Fas system may be central to HSV-tk–mediated cell death.23 In addition, HSV-tk–induced cell death may induce a potent antitumor immunity, which might further improve the efficiency of suicide gene–mediated gene therapy.24-27 In this context, a recent study in murine cell lines demonstrated that the antitumor immunity induced by HSV-tk/GCV was dependent on hsp70 up-regulation during cellular necrosis, whereas apoptotic cell death failed to induce im-
munity. This mechanism may be the result of the potent immunostimulatory effects of heat shock proteins (hsp), which provide an immunologic danger signal and are able to induce and enhance a strong and specific cellular immunity against tumor-associated antigens.

Novel suicide genes such as phosphorylase (PNP) demonstrate more efficient tumor cell killing and enhancement of the bystander effect as compared with HSV-tk. PNP converts certain adenosine analogs into highly toxic metabolites that readily diffuse across cell membranes and interfere with DNA, RNA, and protein synthesis—killing dividing, as well as nondividing, cells. Despite the well-established efficiency of PNP, little is known about the mechanisms underlying cell death and the potential induction of an antitumor immunity. We therefore analyzed the mechanisms of cell death induced by the PNP/fludarabine system in human HCC cells in comparison with HSV-tk.

MATERIALS AND METHODS

Tumor Cell Lines. The human hepatoblastoma cell line, HepG2 (ATCC HB-8065), and the human hepatocellular carcinoma cell line, Hep3B (ATCC HB-8064), were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cell lines were maintained in Dulbecco's modified Eagle medium (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (PA Laboratories, Exton, PA), nonessential amino acids (Gibco), 50 U/mL penicillin G (Gibco), and 50 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air.

Recombinant Adenovirus Vectors. The adenoviral vectors, Ad2/CMV/HSV-tk and Ad2/CMV/PNP, were used for the expression of HSV-tk and PNP in HCC cells. The vectors were generated and purified as described previously.

Adenoviral Transduction and Prodrug Treatment of Cells. GCV (Cytovene, Roche, Nutley, NJ) as prodrug for HSV-tk and fludarabine (Fludara, Berlex, Richmond, CA) as prodrug for PNP were dissolved in sterile water according to the manufacturer's instructions. Cells were trypsinized, infected with Ad2/CMV/HSV-tk or Ad2/CMV/PNP, and seeded in 10-cm tissue culture dishes or 96-well microtiter plates. After 24 hours, the medium was replaced by medium containing the respective prodrugs.

Crystal Violet Assay. Cell viability was determined by crystal violet staining. Cells were grown in 96-well microtiter plates. After removal of the culture medium, cells were washed once with phosphate-buffered saline (PBS), followed by incubation with a solution containing 0.75% crystal violet (Sigma Chemical Co., St. Louis, MO), 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde for 30 minutes at room temperature. Cells were then washed thoroughly with water and air-dried. The dye was then eluted with 200 μL PBS containing 1% sodium dodecyl sulfate (SDS). Cell viability was determined by optical density (OD) measurement at 650 nm on an automated enzyme-linked immunosorbent assay reader. Viability of cells treated with prodrugs was calculated as the mean of quadruplicate OD values divided by the mean of quadruplicate OD values of cells cultured in the absence of prodrug (control cells) and expressed as % of control cells ± SD. The OD obtained for medium alone were subtracted as background.

Sublethal Heat Shock Treatment of Cells. Cellular expression of hsp70 was induced by heat-shock (HS) treatment. For HS treatment, medium of cells cultured in 10-cm tissue culture dishes was replaced by medium at 43°C, and cells were incubated at 43°C for 60 minutes, followed by replacement with medium of a temperature of 37°C. Cells were then incubated at 37°C until immunoblot analysis was performed.

Immunoblot Analysis. Mouse monoclonal antibodies against PARP (C-2-10, Biomol, Plymouth Meeting, PA), p53 (DO-1, Santa Cruz, Santa Cruz, CA), FasL (33, Transduction Laboratories, Lexington, KY), and hsp70 (W27, Santa Cruz, Santa Cruz, CA) were used for Western blot analysis. After prodrug treatment, both adherent and nonadherent cells were collected, washed with PBS, and stored at −80°C until use. Cell lysates were prepared on ice in RIPA lysis buffer (1% NP-40, 0.5% deoxycholate (DOC), 1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 8.0]) containing 10% proteinase inhibitor. Protein concentration was determined using the Protein Assay (Bio Rad, Hercules, CA). Samples containing 50 μg protein were separated on SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Immobilon-P, Millipore, Bedford, MA). Immunoblotting was performed using predetermined optimal dilutions of primary antibodies and horseradish peroxidase–conjugated secondary antibodies (NA9311, Amersham Pharmacia Biotech, Chicago, IL), followed by enhanced chemiluminescence (Super Signal, Pierce, Rockford, IL).

DNA Ladder Assay. Apoptotic fragmentation of DNA was analyzed by detection of the DNA ladder pattern. After prodrug treatment, both adherent and nonadherent cells were collected, washed with PBS, and stored at −80°C until use. Cellular DNA was isolated using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. DNA was analyzed by 1.4% agarose gel electrophoresis, followed by ethidium bromide staining.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay. For in situ detection of DNA fragmentation, cells were cultured on coverslips in 6-well tissue culture plates. After prodrug treatment, nick end labeling was performed using the In-situ Cell Death Detection Kit, AP (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. Labeled cells were stained with Fast Red (Boehringer Mannheim). The coverslips were mounted onto glass slides for light microscopy.

Reverse-Transcriptase Polymerase Chain Reaction Analysis. Cellular expression of Fas ligand (Fasl) was analyzed using the reverse-transcription polymerase chain reaction (RT-PCR) method. Detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA served as a control for the efficiency of the RT-PCR. After prodrug treatment, both adherent and nonadherent cells were collected, washed with PBS, and stored at −80°C until use. Total cellular RNA was purified using the Rnasy Mini Kit (Qiagen) following the manufacturer’s instructions. One microgram of RNA was reverse-transcribed using the 1st-Strand cDNA Synthesis Kit (Clontech, Palo Alto, CA) following the manufacturer’s instructions. cDNA was stored at −80°C until use. For detection of FasL RNA, 10-μL aliquots of the cDNA were amplified in a DNA thermocycler using 2.6 U Taq DNA polymerase (Expand High Fidelity PCR System, Boehringer Mannheim) diluted in the buffer supplied by the manufacturer, in a volume of 50 μL containing all 4 nucleotides in a concentration of 200 μmol/L each of upstream and downstream human FasL primer in a concentration of 200 nmol/L each. The sequence of the human FasL primers used in the experiment has been described previously. PCR was performed in 40 cycles with each cycle consisting of a denaturation step (94°C for 1 minute, 4 minutes for the first cycle only), an annealing step (56°C for 1 minute), and an elongation step (72°C for 1 minute, 8 minutes for the last cycle only). For detection of GAPDH RNA, 5-μL aliquots of cDNA were amplified using upstream and downstream human GAPDH primers (1st-Strand cDNA Synthesis Kit, Clontech) in a concentration of 400 nmol/L each. The PCR was performed in 30 cycles, with each cycle consisting of a denaturation step (94°C for 45 seconds, 4 minutes for the first cycle only), an annealing step (60°C for 45 seconds), and an elongation step (72°C for 2 minutes, 8 minutes for the last cycle only). Each RT-PCR included controls without RNA or cDNA. PCR products were analyzed by 1.4% agarose gel electrophoresis.

Flow Cytometric Analysis. Flow cytometry was used for analysis of annexin V binding and detection of Fas. After prodrug treatment, both adherent and nonadherent cells were collected and washed twice with PBS. The annexin V assay was performed using the annexin V-FITC Apoptosis Detection Kit (PharMingen, San Diego, CA) according to the manufacturer’s instructions. In brief, 105 cells were resuspended in 100 μL binding buffer supplied by the manufacturer and stained with 5 μL annexin V–fluorescein isothiocyanate (FITC).
and 10 µL propidium iodide (PI) for 15 minutes at 4°C in the dark. For Fas detection, 5 × 10⁵ cells were resuspended in 50 µL PBS containing 3% fetal calf serum and 0.1% sodium azide, stained with 20 µL phycoerythrine-conjugated mouse monoclonal antibodies against Fas (DX2, PharMingen) for 15 minutes at 4°C in the dark, and washed with binding buffer. Flow cytometric analysis was performed immediately after the staining procedure using a FACScan Calibur with CellQuest software (Becton Dickinson, San Jose, CA).

**Fas/FasL Blocking Assay.** Neutralizing mouse monoclonal antibodies against Fas (ZB4, Upstate, Lake Placid, NY) and FasL (NOK-1, PharMingen) were employed to block Fas/FasL interaction in HepG2 cells. After transduction of the HSV-tk gene, cells were seeded in 96-well tissue culture plates and incubated with GCV in the presence or absence of 1.0 µg/mL of either antibody. Cell viability was measured using the crystal violet assay as described above.

**RESULTS**

**Induction of Apoptosis.** Apoptosis was assessed by the characteristic cleavage pattern of PARP as determined by immunoblot, agarose gel analysis of DNA fragmentation (DNA ladder) and the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. Cleavage of PARP could be detected in both cell lines expressing PNP or HSV-tk following prodrug administration, indicating that both suicide genes induced apoptotic cell death (Fig. 1A). In HepG2 cells transduced with PNP or HSV-tk, PARP cleavage was observed as early as 24 hours after prodrug administration. In Hep3B cells transduced with PNP or HSV-tk, PARP cleavage was detectable at day 2 and day 4, respectively. PARP cleavage preceded the typical DNA ladder, which was detectable in HepG2 cells at day 2 (PNP/fludarabine) and at day 4 (HSV-tk/GCV), respectively (Fig. 1B). In Hep3B cells, the DNA ladder occurred earlier in cells treated with PNP/fludarabine (day 4) as compared with cells treated with HSV-tk/GCV (day 6). Apoptosis was also demonstrated in situ by the TUNEL assay in both cell lines (Fig. 1C). For quantification of apoptosis, the redistribution of plasma membrane phosphatidylserine by annexin V binding was analyzed (Fig. 2). Similar to PARP cleavage and DNA ladder analysis, apoptosis was detected earlier in HepG2 cells for both prodrug-activating systems as compared with Hep3B cells. In both cell lines, PNP/fludarabine induced apoptosis in a higher percentage of cells as compared with HSV-tk/GCV. At day 4, more than 30% of PNP/fludarabine-transduced HepG2 cells were apoptotic, as compared with 20% in HepG2 cells treated with HSV-tk/GCV. At day 6, approximately 30% of Hep3B cells treated with PNP/fludarabine were apoptotic as compared with 20% in HepG2 cells treated with HSV-tk/GCV. UV irradiation of HepG2 and Hep3B cells was used as control for complete apoptosis. Assessment by annexin V/PI FACS analysis 24 and 48 hours after UV irradiation identified similar numbers of annexin V–positive apoptotic cells (up to 30%) (data not shown). These results indicate that apoptosis is the major mechanism of cell death in HepG2 cells treated with PNP/fludarabine and HSV-tk/GCV, and in Hep3B cells treated with PNP/fludarabine. Hep3B cells treated with HSV-tk/GCV showed a significantly lower percentage of apoptotic cells.

**Induction of p53.** As described above, apoptosis occurred earlier and in a higher percentage of p53-positive HepG2 cells as compared with p53-negative Hep3B cells. To determine
whether p53-mediated apoptosis might represent an important pathway of HSV-tk– or PNP-induced apoptosis, HepG2 cells were analyzed for induction of p53 by Western blot analysis. As shown in Fig. 3, induction of p53 could be observed in HepG2 cells during cell death induced by HSV-tk/GCV and PNP/fludarabine, respectively. Higher levels of p53 were detected following treatment with PNP/fludarabine when compared with HSV-tk/GCV.

Expression of Fas (CD95) and FasL. Cell-surface expression of the Fas receptor (CD95) was determined in HepG2 and Hep3B cells following treatment with PNP/fludarabine or HSV-tk/GCV by flow cytometry. As shown in Fig. 4A, HepG2 cells showed enhanced staining for surface Fas expression following treatment with HSV-tk/GCV, whereas no change in Fas expression could be detected in HepG2 cells treated with PNP/fludarabine and in Hep3B cells following treatment with both prodrug-activating systems. Expression of FasL mRNA was determined by RT-PCR, and FasL protein levels were determined by Western blot. Induction of FasL could be demonstrated in Hep3B and in HepG2 cells following treatment with HSV-tk/GCV, but not following treatment with PNP/fludarabine (Fig. 4B and 4C). To assess the functional importance of the enhanced surface expression of Fas in association with the FasL up-regulation in HSV-tk/GCV–treated HepG2 cells, 2 antibodies (ZB4 and NOK-1) were employed that inhibit Fas/FasL-mediated apoptosis. As shown in Fig. 5, nei-

Fig. 2. Quantification of suicide gene–induced apoptosis. For HepG2 and Hep3B cells transduced with the suicide genes, HSV-tk or PNP, and incubated with the respective prodrugs, the percentage of apoptotic cells was quantified by flow cytometric analysis of annexin V–FITC binding and propidium iodid (PI) uptake. (A) Results are shown for untreated cells and cells at day 4 (HepG2) or day 6 (Hep3B) of prodrug treatment, with gate A representing viable cells; gate B, apoptotic cells; and gate C, cells in an advanced stage of apoptosis or in necrosis. (B) A time-course experiment was performed demonstrating the percentage of apoptotic cells (i.e., % of cells contained within gate [B]) following suicide gene treatment.

Fig. 3. Accumulation of p53 following suicide gene treatment. HepG2 cells were transduced with the suicide genes, HSV-tk or PNP, and incubated with the respective prodrugs for 2, 4, or 6 days. Amounts of p53 were determined by Western blot analysis and quantified by densitometry. The data presented are from 1 representative Western blot of 3 independent experiments.
Expression of hsp70 in Human Carcinoma Cells During Suicide Gene–Induced Cell Death. Expression of hsp70 during HSV-tk/GCV– and PNP/fludarabine-induced cell death was determined by Western blot analysis in HepG2 and Hep3B cells. Whereas a sublethal HS of 1 hour at 43°C resulted in a strong induction of hsp70, no significant increase of hsp70 could be observed during PNP/fludarabine- or HSV-tk/GCV–induced cell death (Fig. 6).

DISCUSSION

In the present study, we examined the mechanisms of cell death induced by the prodrug-activating system, PNP/fludarabine, in comparison with the HSV-tk/GCV system following adenoviral delivery of the respective genes into human HCC cells. Apoptosis has been described previously as the major mechanism of cell death induced by the HSV-tk/GCV system in different tumor cell lines.23,30-42 Our results demonstrate that both prodrug-activating systems induce apoptosis in HepG2 and Hep3B cells, but apoptosis occurred earlier and in a higher percentage of PNP/fludarabine-treated cells as compared with HSV-tk/GCV. These data confirm our previous observations that treatment of HCC cells with the PNP/fludarabine system resulted in a more rapid and more efficient cell death than HSV-tk/GCV.6 Because numbers of apoptotic cells following treatment with PNP/fludarabine approached numbers found after induction of complete apoptosis by UV irradiation, this indicated that PNP/fludarabine can eliminate all treated HCC cells by apoptosis.

Because mutations of the p53 gene are common in human HCC cells, we examined the involvement of p53 in the induction of apoptosis induced by PNP/fludarabine and HSV-tk/GCV, respectively. HepG2 cells expressing wild-type p53 showed a more rapid onset of apoptosis as compared with
p53-negative Hep3B cells following treatment with both prodrug-activating systems, and early accumulation of cellular p53 protein could be detected that was more pronounced following treatment with PNP/fludarabine. Induction of p53 may be caused by the interaction of toxic metabolites with chromosomal DNA. Therefore, p53-mediated apoptosis might be involved in cell death induced by PNP/fludarabine and HSV-tk/GCV. However, after treatment with PNP/fludarabine, p53-negative Hep3B cells showed similar features of apoptotic cell death, and the percentage of apoptotic cells reached similar levels as compared with HepG2 cells. Therefore, PNP/fludarabine-induced apoptosis might involve, but not depend on, p53. In p53-negative Hep3B treated with HSV-tk/GCV, the numbers of apoptotic cells were found to be significantly lower when compared with p53-positive HepG2 cells, which corresponds to the reduced sensitivity of p53-negative Hep3B cells as compared with p53-positive HepG2 cells to treatment with HSV-tk/GCV. This indicates that p53-mediated apoptosis may be significantly involved in the induction of HSV-tk/GCV–induced cell death. However, it cannot be excluded that the HSV-tk/GCV system also induces to some extent nonapoptotic cell death in human HCC cells that has been previously described for rat hepatoma cells and murine melanoma cells or by an irreversible G2-M arrest.

Because cellular p53 accumulation induces Fas-mediated apoptosis by transcriptional activation of the Fas gene and by cell-surface trafficking of Fas, we analyzed Fas expression on the cell surface of p53-positive HepG2 and p53-negative Hep3B cells, as well as the induction of Fasl following treatment with HSV-tk/GCV and PNP/fludarabine, respectively. HepG2 cells showed p53 accumulation and were more sensitive to HSV-tk/GCV–induced apoptosis as compared with p53-negative Hep3B cells. As reported previously for apoptosis induced by bleomycin and other chemotherapeutic drugs, we found enhanced Fas expression at the cell surface only in p53-positive HepG2 cells. Furthermore, we observed a strong induction of Fasl mRNA and Fasl protein in both cell lines following treatment with HSV-tk/GCV, which has also been observed in p53-positive, p53-negative, and p53 mutant HCC cell lines after treatment with various anticancer drugs. Our findings therefore suggest that the Fas/Fasl pathway could be involved in HSV-tk/GCV–mediated cell death in human HCC cells similar to various murine cell lines.

To analyze the extent of Fas/Fasl-mediated apoptosis in HSV-tk/GCV–induced cell death in human HCC cells, we used neutralizing mouse monoclonal antibodies against Fas (ZB4) and Fasl (NOK-1) that were shown to inhibit HSV-tk/GCV–mediated apoptosis in murine tumor cells. Interestingly, there was no inhibition of apoptosis in HSV-tk/GCV–treated HepG2 cells following incubation with both neutralizing antibodies. While treatment with HSV-tk/GCV results in p53 accumulation and increased expression of both Fas and Fasl in p53-positive HepG2 cells, the Fas/Fasl pathway does not appear to be of major functional importance in HSV-tk/GCV–induced apoptosis in these cells. These findings are different from results in murine cells in which p53 accumulation and induction of apoptosis via the Fas/Fasl pathway has been described following treatment with HSV-tk/GCV. These differences may be explained by cell line–specific differences of HSV-tk/GCV–mediated apoptosis or by experimental differences. We used adenoviral HSV-tk gene transfer at a relatively high multiplicity of infection and expressed the transgene under the transcriptional control of the cytomegalovirus promoter, which may result in higher HSV-tk expression levels than that of the stably HSV-tk–expressing cell lines used by Wei et al. In combination with the higher concentrations of GCV used in our study, this may have resulted in more rapid and stronger synthesis of toxic metabolites in our HSV-tk/GCV–treated HepG2 cells, which may have induced apoptosis mainly through pathways not involving the Fas/Fasl, signaling or even cell death by necrosis in a proportion of cells.

In contrast, following treatment with PNP/fludarabine, we observed no changes in cell-surface Fas expression despite a strong p53 accumulation in HepG2 cells. Furthermore, there was no up-regulation of Fasl mRNA and Fasl protein in HepG2 and Hep3B cells. Our data demonstrate that Fas-mediated apoptosis is not involved in apoptosis induced by PNP/fludarabine in human HCC cells. This may be partially explained by the potential inhibition of RNA and protein synthesis by toxic metabolites induced by PNP, which could interfere with the up-regulation of Fasl. and with the p53-mediated increased expression of the Fas gene. Because p53 up-regulation is mainly the result of stabilization of the p53 protein, and not of de novo protein synthesis, this could explain the observed increase in p53 following treatment with PNP/fludarabine despite potential inhibition of protein synthesis.
The regression of tumors via the HSV-tk/GCV system in vivo seems not only to be mediated by the pharmacologic bystander effect, but also seems to involve immune mechanisms.\(^4\)\(^,\)\(^2\)\(^,\)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\) Furthermore, there is growing evidence that the induction of necrosis by suicide gene therapy may induce a stronger tumor-specific immunoreactivity than apoptosis. This may be caused by the induction of danger signals for the immune system such as up-regulation of hsp.\(^2\)\(^,\)\(^2\)\(^9\) Increased hsp expression results in enhanced presentation of tumor antigens via major histocompatibility complex class I molecules on the cell surface of tumor cells. Furthermore, uptake of hsp-peptide complexes by antigen-presenting cells leads to efficient presentation of tumor antigens to tumor-specific T cells, and hsp uptake induces cytokine release by polymorph nuclear cells.\(^2\)\(^9\) It could be demonstrated that the immunostimulatory effects of HSV-tk/GCV–mediated tumor treatment depends on the induction of hsp70 in the treated tumors, resulting in the release of TH1 cytokines and enhanced antigen uptake by immature dendritic cells.\(^2\)\(^8\)\(^,\)\(^3\)\(^2\) We were interested to see whether the hsp70 induction observed in murine tumor cell lines following treatment with HSV-tk/GCV could also be demonstrated in human HCC cell lines during suicide gene–mediated cell death and whether there was a difference between the 2 suicide-gene systems used. No significant increase of hsp70 during cell death induced by HSV-tk/GCV or by PNP/fludarabine could be demonstrated. Our results support the observation by Melcher et al.\(^2\)\(^8\) that hsp induction does not occur during apoptotic cell death following suicide gene therapy. These findings indicate that the induction of an antitumor immune response by treatment with HSV-tk or PNP/fludarabine might be limited. However, further studies are needed to elucidate whether the induction of an antitumor immune response by suicide gene therapy depends on hsp induction, or whether the enhanced uptake of apoptotic tumor cells by dendritic cells may be sufficient to induce a cellular antitumor immune response.\(^3\)\(^0\)\(^,\)\(^3\)\(^1\)\(^,\)\(^3\)\(^2\)\(^,\)\(^3\)\(^3\)

In summary, our data demonstrate that both HSV-tk and PNP/fludarabine induce cell death predominantly by apoptosis in human HCC cells. Using the HSV-tk/GCV system, apoptosis was induced more efficiently in p53-positive cells as compared with p53-negative cells. Despite activation of the Fas/FasL pathway, we demonstrated that the Fas/FasL system is not significantly involved in HSV-tk/GCV–induced apoptosis in p53-positive HepG2 cells. In comparison, apoptosis induced by the PNP/fludarabine system in p53-positive cells occurs earlier and may to some extent involve p53, but efficiency of PNP/fludarabine-induced apoptosis is similar both in p53-positive and p53-negative cells, and is independent of the Fas/FasL signaling pathway. This may be advantageous for gene therapy of tumors carrying p53 mutations or which are resistant to Fas mediated apoptosis. In addition, both prodrug-activating systems failed to induce up-regulation of hsp70 in HCC cells, which might limit the efficiency of these prodrug-activating systems to induce a potent antitumor immune response against HCC.

REFERENCES

22. Wei SJ, Chao Y, Shih YL, Yang DM, Hung YM, Yang WK. Involvement of Fas (CD95/APO-1) and Fas ligand in apoptosis induced by ganciclovir treatment of tumor cells transduced with herpes simplex virus thymidine kinase. Gene Ther 1999;6:420-431.


