Combine cancer gene therapy harnessing plasmids expressing human tumor necrosis factor alpha and Herpes simplex thymidine kinase suicide gene*

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Received March 16, 2006

We have assessed the effect of combine cancer gene therapy with exogenous human tumor necrosis factor alpha (*hTNFα*) and suicide gene therapy on three human cancer cell lines MCF-7 (breast adenocarcinoma), U-118 MG and 42-MG-BA (human gliomas). Transfection of a plasmid containing *hTNFα* under the control of a hybrid promoter resulted in expression of *hTNFα* gene *in vitro.* Transduction of retroviral plasmid containing *Herpes simplex* thymidine kinase (*HSVtk*) led to the expression of thymidine kinase in all three cell lines. MTT cell proliferation assay and flow cytometric analysis showed a significant increase in apoptotic and necrotic cells and decrease of proliferation in all cell lines after combine therapy with *hTNFα* expression plus thymidine kinase/GCV suicide system. The presence of these two genes after transduction of retroviral vector containing thymidine kinase and *hTNFα* was confirmed by PCR. The expression of *HSVtk* gene was proved by Western blot analysis, and the expression of both genes was confirmed by RT-PCR. Additive cell killing effect due to presence of *HSVtk* and *hTNFα* therapeutic genes after activation of non-toxic prodrug was observed. Whether the bicistronic plasmid containing both genes would improve the therapeutic effect need to be assessed in the future.

Key words: cancer gene therapy, retroviral vector, Herpes simplex thymidine kinase, tumor necrosis factor alpha, apoptosis

Suicide gene therapy using the *Herpes simplex* thymidine kinase (*HSVtk*)/ganciclovir (GCV) has been considered as a promising cancer therapy. The basic principle underlying metabolic suicide gene systems is intracellular conversion of relatively non-toxic prodrug to a toxic drug by an enzyme that is absent in the cell. Suicide genes currently under investigation mediate sensitivity by encoding viral (*Herpes simplex* virus thymidine kinase, *Varicella zoster* virus thymidine kinase), bacterial (*E. coli* cytosine deaminase, purine nucleoside phosphorylase, uracil phosphoribosyl transferase, nitroreductase, carboxypeptidase G2, xanthine-guanine phosphoribosyl transferase) or mammalian (carboxylesterase, cytochrome P-450 2B1, deoxycytidine kinase, thymidine phosphorylase) enzymes [1–17]. One of the well-characterized activating enzymes is the *Herpes simplex* virus thymidine kinase (*HSVtk*) [18], which phosphorylates antiviral nucleoside analogues such as ganciclovir to ganciclovir monophosphate. Ganciclovir phosphate is further phosphorylated by cellular kinases to ganciclovir triphosphate, which inhibits DNA polymerase and incorporates into DNA in place of guanosine-5'-triphosphate, thus leading to the inhibition of chain elongation. An important component of this therapeutic approach is phenomena called bystander effect – the ability to induce the death of adjacent tumor cells not expressing *TK* gene [19–21]. As for *HSVtk*/GCV system many *in vitro* bystander killing assays have revealed that a tumor cell transduction rate of 10 to 20 percent or even less resulted in significant tumor reduction. In experiments *in vitro* using several glioma cell lines it was reported that at a transduction rate 5 percent only, the killing effect reached 90 percent [19]. For the efficiency of bystander effect, the importance of cell-cell contacts between parental and *HSVtk* transduced cells, realized through gap junction intercellular communication (GJIC), was found important [7]. The phenomenon of the bystander effect is not fully understood. Except of GJIC there are some other hypothetic mechanisms, which should be involved *in vivo* killing of non-transduced cells. One could be the released apoptotic factors from dead and dying cells

*The study was supported by the VEGA grants No. 2/3094/24, 2/3094/25, 6061/26, and by state program "Use of cancer genomics to improve the human population health".

[20, 22]. The second possibility is also killing of capillary endothelial cells, which had been *TK*-transduced and died after GCV treatment, thus reducing the nourishment of the tumor [23]. The stimulation of an immune response could be considered as well [24–26]. It is likely that all mentioned hypothetic mechanisms of bystander effect take place *in vivo*. A crucial role in cancer gene therapy is the transduction of suicide gene into as many tumor cells as it is possible. Insufficient bystander effect observed *in vivo* is likely due to inadequate infection of tumor mass, despite that the infection was performed with high titer virus producing cells [27–31]. In order to improve killing effect of suicide gene therapy within the cancer cells expressing *HSVtk* we decided to try to multiply the effect by transfection of plasmid expressing the human tumor necrosis factor (*hTNFα*). Although the molecular mechanism of *TK*/GCV cytotoxicity is poorly understood, there is a strong evidence of apoptosis induction both in p53 deficient and p53 positive cells. BELTINGER showed that TK/GCV treatment lead to accumulation of p53 protein in p53 positive cells [32]. Accumulation of p53 caused cell surface expression of CD95 and tumor necrosis factor receptor, which then lead to p53-mediated translocation of CD95 associated with CD95-L-independent formation of death inducing signal and caspase 8 activation [32]. P53 deficient cells after incorporation of GCV triphosphate into DNA resulted in replication-dependent formation of double-strand DNA breaks. Consequently the cells were arrested in S and G2/M phases with activation of caspase -9/-3 [33].

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine that is secreted by macrophages and can elicit a variety of effects. Human TNFα is known to be cytotoxic to variety of human cancer cells *in vitro* [34] and its cytotoxicity has been observed in human tumor bearing mice where hemorrhagic tumor necrosis was induced after intratumoral TNFα delivery [35–38]. Because of its toxicity the use of recombinant hTNFα in systemic cancer therapy is rather limited. The mechanism, by which hTNFα leads to cell death is complex. It is dependent on the presence both of cell-surface receptors and other proteins present in the cells, such as pro-apoptotic and anti-apoptotic factors, on the cell cycle phase, and on other unknown factors. The hTNFα is initially expressed as a 26 kDa membrane bound protein, which is cleaved by the hTNFα converting enzyme that generates the soluble 17 kDa active protein. In the circulation, monomers of hTNFα form homotrimers. The induction of various cellular responses mediated by hTNFα is initiated by its interaction with two distinct cell surface receptors, tumor necrosis factor receptors TNFR1 and TNFR2 that are independently expressed on different cell types [39, 40]. While TNFR1 and TNFR2 each possess a typical TNFR structure, the receptors show little amino acid identity. TNFR1 contains a cytoplasmic death domain, which is important in initiating apoptosis and other signaling pathways after hTNFα binding. The death domain-containing receptor is maintained in an inactive state by a 60 kDa protein, named silencer of death domain. Binding of hTNF-α to its receptor results in receptor trimerisation and aggregation of the death domain. This allows binding of an intracellular adapter molecule TNFR-Associated Death Domain (TRADD), which interacts with the death domain and subsequently, binds various proteins to the activated receptor, thus directing the future fate of the cell. The association of TRADD with Fas-Associated Death Domain (FADD) leads to the induction of apoptosis *via* the recruitment and cleavage of pro-caspase 8 [41, 42]. Caspase 8 activation provides a link between death receptor signaling (extrinsic) and the mitochondrial (intrinsic) pathways of the apoptosis [43–46]. Apoptosis can be also mediated through the interaction between TNFR1 and an adapter molecule designated as Receptor-Interacting Protein RIP-Associated ICH-1 CED-3 homologous protein with death domain (RAIDD). RAIDD associates with RIP through interaction between death domains, recruits caspase 2 and leads to the induction of apoptosis [47]. TNFR2 receptor is involved in signaling the proliferation of thymocytes, cytotoxic T cells and human mononuclear cells [48]. Although each receptor can individually mediate *TNFα* activity, some physiological activities often require the presence and interaction of both receptors [49–51].

In the present study, we have investigated the effectiveness of the combine cancer gene therapy using suicide system *HSVtk*/GCV with expression of *hTNFα*. The presence of enhanced killing effect on human cancer cell lines was proved by the measurement of cell proliferation and apoptosis. Transfection of hTNFα expression vector into human tumor cells contaning *HSVtk* gene lead to increased cell death.

Material and methods

Expression vectors. pORFhTNFα (InvivoGen, San Diego, USA) contains an intron-less open reading frame of the human *TNFα* downstream of the EF-1α/HTLV hybrid promoter. The control plasmid pORF-minus-hTNFα was constructed as described previously by digesting pORF-hTNFα with SgrA I and Nhe I to remove the hTNFα fragment [52]. Schematic maps of the plasmids are presented in Figure 1. Green fluorescent protein expression plasmid pCIneoGFP was used for determination of the transfection efficiency (kindly provided by Geoff Margison and Joanna Libby, Paterson Institute for Cancer Research, Christie Hospital, Manchester).

Retroviral vectors. pJH5 bicistronic retroviral vector containing Herpes simplex thymidine kinase and gene for neomycin resistance separated by IRES sequence, both genes expression are under the LTR promoter/enhancer region [2].

Schematic maps of the plasmids are presented in Figure 1.

Tumor cell lines. The following cell lines were used: MCF-7 (human breast adenocarcinoma), U-118 MG (human glioma) and 42-MG-BA (human glioma). The glioma cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum,

100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 in air. The MCF-7 cell line was cultured in RPMI supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Cell transfection. 10^5 cells were transiently transfected with 1 µg of either pORF-hTNFα or pORF-minus-hTNFα using GeneJuice transfection reagent (Novagen) or Effectene (Qiagen) according to the manufacturer's recommendation. Plasmid pCINeoGFP expressing green fluorescent protein was used to optimize the ratio between amount of DNA and transfection reagent. The transfection medium was replaced with fresh medium after 14 hours.

Retroviral infection of cancer cell lines. One day before infection, $2x10^5$ recipient cells were plated in 60 mm Petri dishes. For transduction of U-118 MG, 42-MG-BA, and MCF-7 cells medium filtered through a 0.45 µm filter from retrovirus producing cells PA317cl13pJH5cl13 [2] was used. Infection was done by replacing the medium of the recipient cells with 2 ml of medium having viral particles supplemented with Polybrene 8 µg/ml. Selection medium contained G418 was applied twenty four hours after virus infection. The resistant cell lines U-118 MG/TK, 42-MG-BA/TK, MCF-7/TK were obtained after 14 days of G418 selection.

Analysis of hTNFα gene and TK gene presence by PCR. Total cellular DNA from transfected cells was obtained by phenol/chloroform extraction. The specific primers for PCR detection of *hTNFα* and *TK* were used to confirm the presence of the transgene.

Nucleotide sequence of primers:

TNF forward:
5'CCGGAATTCACCATGAGCACTGAAAGCATG 3'
TNF reverse:
5'ATAGTTTAGCGGCCGCGAATTCCTCACAGGGCA ATG 3'
hTNFalpha 702bp fragment
TK forward:
5'ATTGGATCCCTTGTAGAAGCGCGTATGG 3'
TK reverse:
5'CACGTCGACTCAGTTAGCCTCCCCCATCTC 3'
TK 1200bp fragment

Analysis of hTNF alpha and HSV thymidine kinase expression by RT-PCR. Total cellular RNA was isolated by using Trizol reagent preparation protocol. RNA was DNAase treated using RNeasy Mini Qiagen kit. The lack of DNA contamination in cleaned RNA samples was confirmed by PCR. RT reaction was carried out at 42 °C for 60 minutes and a cooling step at 5 °C for 5 minutes with 1 µg of RNA and random hexamer primers supplied with the kit. Obtained cDNA was used for PCR reactions with *TNFα*, *HSVtk* specific primers. *GAPDH* primers were used as an internal control.

Sequence of primers:

GAPDH forward: 5'GAAGGTGAAGGTCGGAGTC 3'
GAPDH reverse: 5'GAAGATGGTGATGGGATTTC 3'
GAPDH 226bp fragment
TNF forward: 5'CAGAGGGAAGAGTTCCCCAG 3'
TNF reverse: 5'CCTTGGTCTGGTAGGAGACG 3'
TNF 326bp fragment
TK forward: 5'GGAGGACAACACATCGACCG 3'
TK reverse: 5' GCAGATACCGCACCGTATTGGC3'
TK 122bp fragment

Analysis of HSV thymidine kinase expression by Western blot analysis.

Protein cell extracts were prepared by lysis solution (1% SDS, 10 mmol Tris HCl, pH 7.5, 1 mmol PMSF, 1% protease inhibitor cocktail Sigma). The sonicated extract was centrifuged at 20 000 x g for 30 minutes at 4 °C. The protein concentration was determined by Lowry protein assay. Cell extract was separated by 12.5% SDS-PAGE electrophoresis, blotted to nitrocellulose membrane and HSV thymidine kinase presence was proofed by rabbit polyclonal serum directed against *HSVtk* (kindly provided by Dr. J. Pastorek, Institute of Virology, Bratislava). After binding of the second antibody anti-rabbit-AP conjugate the blot was visualized using WesternBlue™ stabilized substrate (Promega). As a protein loading control, the same amount of proteins separated by 12.5% SDS-PAGE electrophoresis were stained with Coomassie Brilliant Blue R-250 (Biorad) and distained with methanol-water-acetic acid mix with agitation.

Biological effects of hTNF and TK/GCV therapy in transfected cells. Apoptotic and necrotic cells were quantified after transfection with plasmids and cultivation with or without addition of ganciclovir by flow cytometry (EPICS ALTRA) using the Annexin V-FITC kit (Immunotech) according to the manufacturer's recommendation. In addition the MTT assay was conducted to determine the cell survival rate. Fourteen hours after transfection, 10^4 cells per well were plated into 96-well plates, 6 hours later cells were treated with different concentrations of GCV (Roche) and continued to be cultured for five days, when the me-

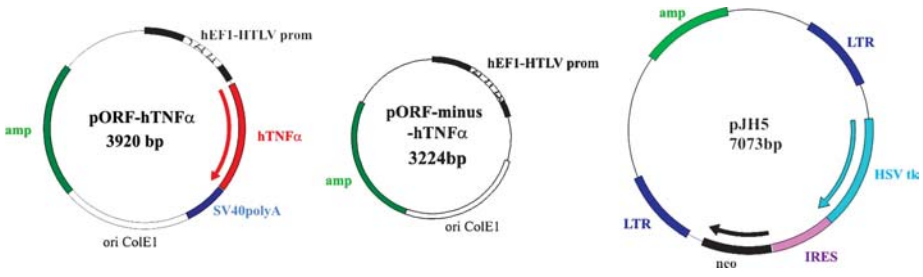

Figure 1. Schematic maps of expression plasmid vectors pORFhTNFα, control plasmid pORF-minus-hTNFα and retroviral vector pJH5.

dium was replaced by MTT mixture. The absorbance of the cells was measured after 3 hours at a wavelength of 490 nm using an ELISA plate reader. The survival rate was expressed as A/B x 100% minus background, where A is the absorbance value from the experimental cells and B is absorbance of the control cells (U-118 MG/TK, 42-MG-BA/TK, MCF-7/TK transfected with control plasmid pORF-minus-hTNF without prodrug treatment).

Statistical analysis. Quantitative variables are described as mean ± standard error of the mean, both expressed as percentages. Comparison between percentages was performed with the Student's t-test. A p value <0.05 was taken as the criterion of statistical significance.

Results

In order to study combine effect of HSV thymidine kinase suicide gene with action of exogenous human tumor necrosis factor alpha (*hTNFα*) the MCF-7 (human breast adenocarcinoma) cells, U-118 MG and 42-MG-BA (human gliomas) cells containing integrated HSV thymidine kinase suicide gene were transfected with expression *TNFα* gene plasmid. The presence of those two genes was confirmed by PCR assay (Fig. 2).

The expression of *HSVtk* gene was found similar by RT-PCR assay in all tested *HSVtk* transduced cells transfected with control plasmid and also in all cells transfected with pORF-hTNF. U-118 MG/TK+pORF-hTNFα reached the highest level of *hTNFα* gene expression (Fig. 3).

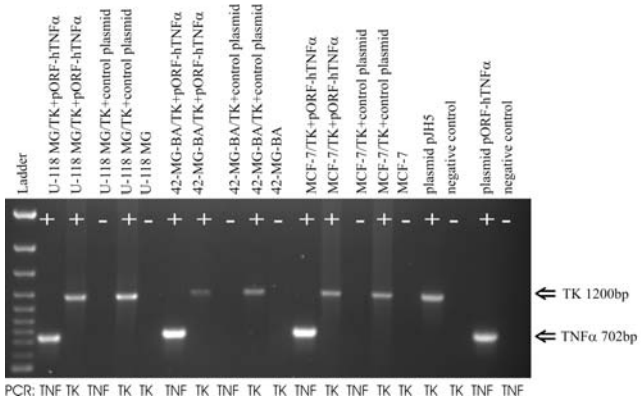

Figure 2. Detection of exogenous *hTNFα* gene and *Herpes simplex* thymidine kinase gene in transfected and transduced cells by PCR. Lane 1 – DNA ladder; lanes 2, 3 – U-118 MG/TK cells transfected with pORF-hTNFα; lanes 4, 5 – U-118 MG/TK transfected with control plasmid pORF-minus-hTNFα; lane 6 – U-118 MG; lanes 7, 8 – 42-MG-BA/TK cells transfected with pORF-hTNFα; lanes 9, 10 – 42-MG-BA/TK cells transfected with control plasmid; lane 11 – 42-MG-BA; lanes 12, 13 – MCF-7/TK cells transfected with pORF-hTNFα; lanes 14, 15 – MCF-7/TK transfected with control plasmid; lane 16 – MCF-7; lane 17 – PCR reaction with pJH5 plasmid; lane 18 – PCR reaction mixture without DNA; lane 19 – PCR reaction with pORF-hTNFα plasmid; lane 20 – PCR reaction mixture without DNA.

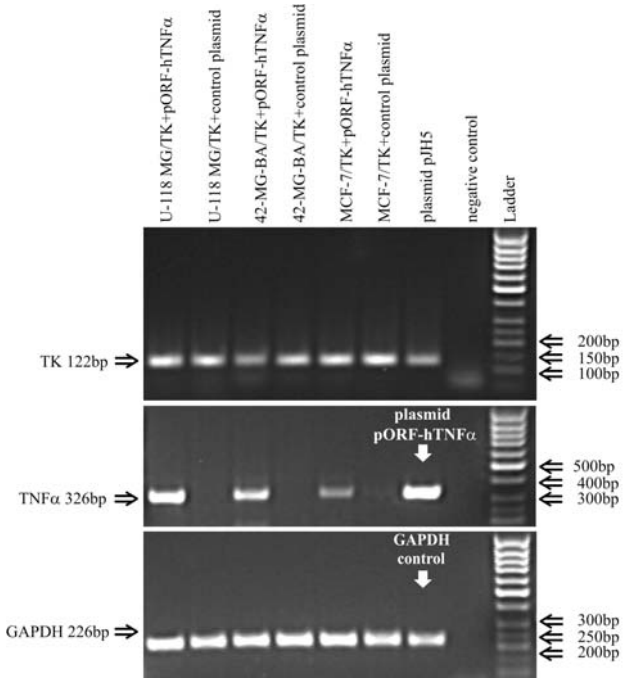

Figure 3. Detection of exogenous *hTNFα* and *Herpes simplex* thymidine kinase expression in transfected and transduced cells by RT-PCR. In order U-118 MG/TK cells transfected with pORF-hTNFα, U-118 MG/TK cells transfected with control plasmid pORF-minus-hTNFα, 42-MG-BA/TK cells transfected with pORF-hTNFα, 42-MG-BA/TK cells transfected with control plasmid, MCF-7/TK cells transfected with pORF-hTNFα, MCF-7/TK cells transfected with control plasmid, RT-PCR control reaction with pJH5 or pORF-hTNFα plasmid or GAPDH cDNA, RT-PCR reaction mixture without cDNA.

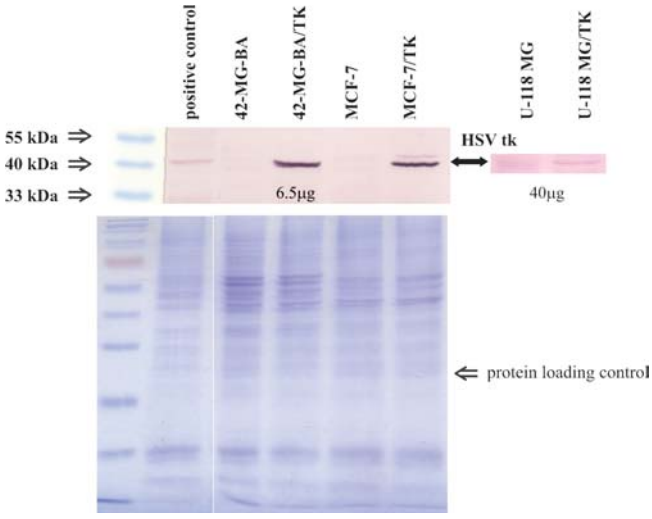

Figure 4. Detection of HSV thymidine kinase protein in total cell lysates. The lysates of transduced cells were analyzed by SDS-PAGE electrophoresis, blotted and detected by rabbit polyclonal antibody against *HSVtk*. 6.5 µg of total proteins from control cell – PA317cl13pJH5cl13, 42-MG-BA, 42-MG-BA/TK, MCF-7, MCF-7/TK and 40 µg of U-118 MG, U-118 MG/TK were separated.

Western blot analysis revealed high level of HSV thymidine kinase protein expression in 42-MG-BA/TK and MCF-7/TK cells, but the low expression of this protein was detectable using more than 6 fold higher concentration of the U-118 MG protein lysate (Fig. 4).

Transfection effectiveness was monitored by flow cytometric estimation of percentage of GFP positive cells after transfection with GFP expressing plasmid. The transfection efficiencies was estimated to be 39.3±0.3% for the U-118 MG/TK cells, 3.9±0.1% for the 42-MG-BA/TK cells, and 50.43±0.9% for the MCF-7/TK cells.

The MTT assay was used to determine the cell survival rate. The survival rate of *TK* gene containing cells was significantly lower in a dose GCV-dependent manner ($p<0.05$––0.001) than in corresponding parental cells (U-118 MG, 42-MG-BA, MCF-7). The cells expressing both HSV thymidine kinase and *TNFα* gene (U-118 MG/TK+hTNFα, 42-MG-BA/TK+hTNFα, MCF-7/TK+hTNFα) were shown the most sensitive to GCV with a half lethal dose significantly decreased. The *TNFα* gene expression in U-118 MG/TK+hTNFα cells lead to 31.8% cell survival as such, whereas for the U-118 MG/TK cells the half lethal dose was 194.1 μg/ml of GCV (Fig. 5). GCV half lethal dose for 42-MG-BA/TK+hTNFα was found 0.00015 μg/ml, for 42-MG-BA/TK cells 0.00823 μg/ml, which was 56 times higher (Fig. 6). Similarly the MCF-7/TK+hTNFα cells were 15.5 times more GCV-sensitive, with the half lethal dose being 0.064 μg/ml, in comparison with 0.993 μg/ml corresponding to MCF-7/TK cells (Fig. 7). The survival rate of parental U-118 MG, 42-MG-BA, and MCF-7 cells was not affected by the presence of GCV.

To quantify the apoptotic portion of the treated cells by flow cytometry, annexin V/PI dual staining was performed measuring the cells 72 or 144 hours after transfection in all variations of treatments (after single either *TK*/GCV or *TNFα* gene treatment; after combine *TK*/GCV plus *TNFα* treatment). The percentage of apoptotic cells in U-118 MG/TK cell line with GCV addition in comparison with U-118 MG/TK without GCV treatment was significantly increased ($p<0.05$). There was additional increase ($p<0.05$) in number of apoptotic cells when U-118 MG/TK cells were transfected with *TNFα* containing plasmid and treated with

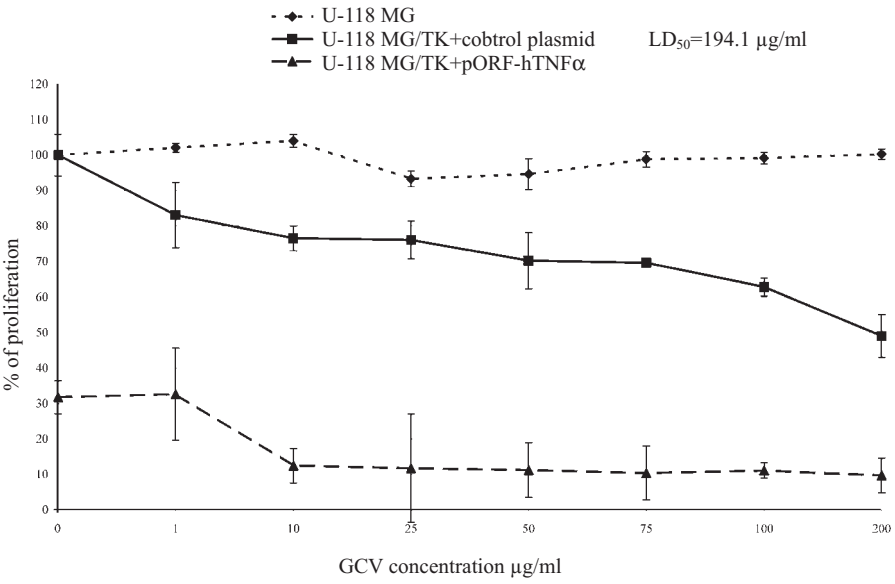

Figure 5. Dose-dependent 5-day survival of U-118 MG cells, U-118 MG/TK cells transfected with control plasmid pORF-minus-hTNFα, and U-118 MG/TK cells transfected with pORF-hTNFα cultured in different concentration of ganciclovir. Percentage of proliferating cells was detected by MTT assay.

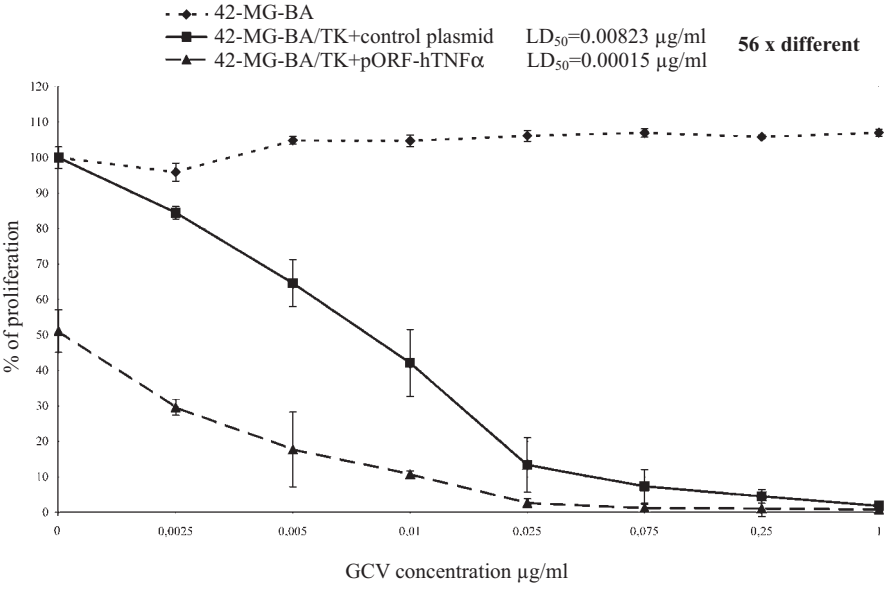

Figure 6. Dose-dependent 5-day survival of 42-MG-BA cells, 42-MG-BA/TK cells transfected with control plasmid pORF-minus-hTNFα, and 42-MG-BA/TK cells transfected with pORF-hTNFα cultured in different concentration of ganciclovir. Percentage of proliferating cells was detected by MTT assay.

GCV. The proportion of living cells decreased after single GCV treatment using two different concentrations of GCV in U-118 MG/TK cells, but was more intense when human *TNFα* was expressed as well. However, there was no difference in cell survival between U-118 MG/TK cells and U-118 MG/TK transfected with pORFhTNFα without GCV treatment (Tab. 1, Fig. 8). The enlarged amount of apoptotic cells appeared already 72 hours after pORFhTNFα transfection in all treated cells, being more pronounced 144 hours following the transfection.

The 42-MG-BA/TK cells were less sensitive to single GCV-treatment, however they possessed much higher sensitivity to *TNFα* expression alone (Tab. 2, Fig. 9). The percentage of apoptotic cells in 42-MG-BA/TK cell line without GCV was 14.2% and 48.7% in 42-MG-BA/TK *TNFα*-expressing cells without GCV treatment. Single GCV-treatment of 42-MG-BA/TK cells with 0.0009 μg/ml or 0.0018 μg/ml lead to apoptosis in the range of 22–25 percent. In combine treatment of cells (42MG-BA/TK plus *TNFα*) in the same GCV concentration rate the amount of apoptotic cells increased to 51.4% and 56.8% respectively. There was no significant difference in proportion of alive, apoptotic, and necrotic cells in 42-MG-BA/TK cell line if we compare period of time 72 hours and 144 hours after pORF-hTNFα transfection (Tab. 2, Fig. 9).

Both the MTT and flow cytometry results of combined suicide gene therapy (*HSVtk*/GCV with expression of *hTNFα*) demonstrated the enhanced killing effects to various human tumor cells.

Discussion

Despite advances in surgical, radiation, chemotherapy, and gene therapies, the prognosis for patients with malignant glioma and other brain tumors with diffuse and invasive character remains grim. A purely surgical cure cannot be achieved because of the infiltrative nature of the tumor into adjacent functioning brain. Radiation therapy is generally used but is unsatisfactory. This study demonstrated that simultaneous prodrug activation by *HSVtk* and expression of *TNFα* in different human cancer cell lines yields an anticancer effect that is enhanced compared with the effect observed with single-prodrug treatment or cytokine expression. Cancer gene therapy with *HSVtk*

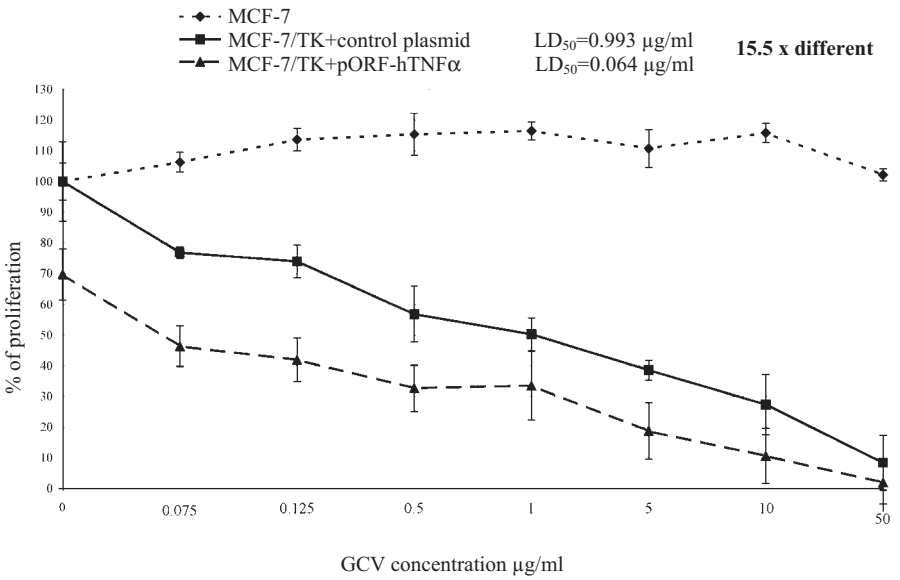

Figure 7. Dose-dependent 5-day survival of MCF-7 cells, MCF-7/TK cells transfected with control plasmid pORF-minus-hTNFα, and MCF-7/TK cells transfected with pORF-hTNFα cultured in different concentration of ganciclovir. Percentage of proliferating cells was detected by MTT assay.

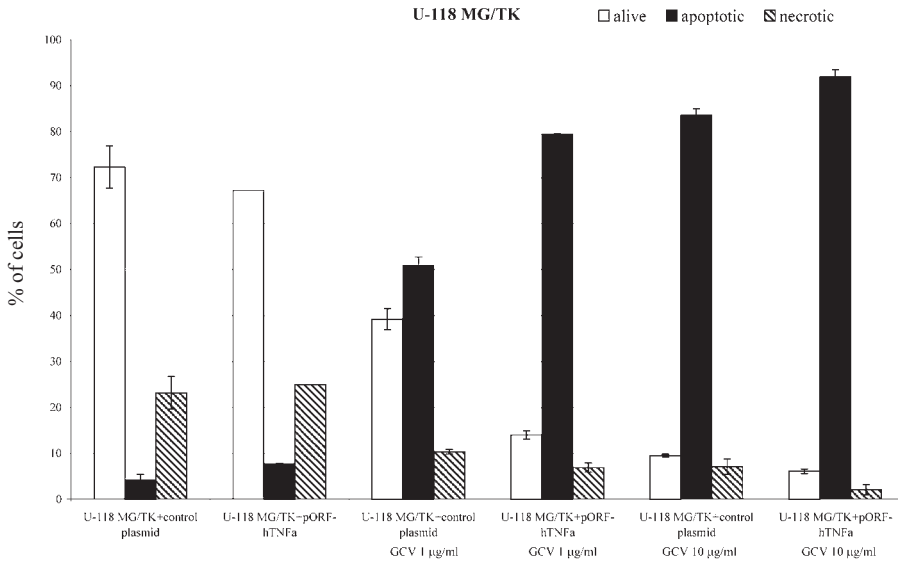

Figure 8. Induction of apoptosis by treatment of U-118 MG/TK cells transfected with control plasmid pORF-minus-hTNFα, and U-118 MG/TK cells transfected with pORF-hTNFα with GCV. Double annexin-V-FITC and PI staining of *HSVtk* transduced and 5-days GCV treated U-118 MG cells revealed the increased apoptotic portion of cells, which was enlarged after hTNFα action.

combined with GCV treatment offers two attractive features for the eradication of dispersed tumor cells in brain. Firstly, because of its specificity for dividing cells *HSVtk*/GCV therapy offers the potential to destroy dispersed tumor cells without harming the tumor surrounding normal tissues. Secondly, non-transduced tumor cells can be eliminated by means of bystander effect involving transmission of TK-activated GCV across gap junction. In addition another benefit for malignant glioma treatment is the preference of retrovirus vectors to transduce actively dividing cells.

Similar results were reported [53] showing that *TNFalpha* expression from *HSVtk* carrying vector potentiated the killing effect of both TNFalpha-sensitive murine fibroblast-like tumor cells L929 and TNFalpha-resistant human glioblastoma cells U-87MG *in vitro*. Prolonged survival of nude mice with brain implant of U-87MG human glioblastoma cells was obtained with combined *HSVtk* suicide gene therapy with vector-based *TNF* expression. The portion of tumor-free animals was also increased with dual therapy [53,54] demonstrated that codelivery of *HSVtk* and *TNFα* followed by GCV treatment was more effective therapy for experimental fibrosarcomas than single *HSVtk* strategy. On the other hand NIRANJAN [55] showed just marginally improved results by combining *TNFα* gene therapy with *HSVtk*/GCV system for 9L intracranial tumor-bearing rats. ZHANG [56] revealed just slightly lower growth but without significant difference of human gastric cancer SGC7901 when expressing *HSVtk* and *TNFα*.

The efficiency of *TNFα* gene therapy alone was found dependent on type of tumor cells used for the treatment [52, 57–59]. Obviously the combined *HSVtk* and *TNFα* therapy are dependent on the kind of tumor cells used for the treatment as well. In our experiments double-gene strategy combining enhanced suicide gene therapy with *TNFα* cytokine expression was found

Table 1. The proportion of alive, apoptotic, and necrotic U118-MG/TK cells 72 or 144 hours after transfection and addition of GCV

GCV/μg/ml Hours after transfection	Control plasmid*			pORF-hTNFα*		
	Alive %	Apoptotic %	Necrotic %	Alive %	Apoptotic %	Necrotic %
0 – 72 hours	81.4	12.54	6.06	79.65	15.72	4.63
0 – 144 hours	72.3±4.6	4.2±1.2	23.2±3.5	67.2±0	7.73±0.1	25±0
1 – 72 hours	51.49	36.34	12.16	41.19	49.65	6.12
1 – 144 hours	39.2±2.3	50.8±1.9	10.33±0.5	14±0.9	79.4±0.2	6.9±1
10 – 72 hours	17.42	60.89	21.65	8.37	71.58	19.98
10 – 144 hours	9.5±0.3	83.6±1.4	7.1±1.7	6.1±0.5	91.9±1.6	2.1±1.1

*The efficiency of transfection measured by GFP expression was 39.3±0.3%

Table 2. The proportion of alive, apoptotic, and necrotic 42-MG-BA/TK cells 72 or 144 hours after transfection and addition of GCV

GCV/μg/ml Hours after transfection	Control plasmid*			pORF-hTNFα*		
	Alive %	Apoptotic %	Necrotic %	Alive %	Apoptotic %	Necrotic %
0 – 72 hours	65	23.2	11.8	33.4	46.1	20.5
0 – 144 hours	75.8±5.2	14.2±3.8	9.9±1.5	34.4±0.1	48.7±1.1	16.8±1
0.0009 – 72 hours	66.8	19.9	13.4	30.6	46.9	22.5
0.0009 – 144 hours	66.4±0.6	22±1.1	11.5±1.7	30.1±0.2	51.4±0.3	18.3±0.4
0.0018 – 72 hours	67.92	21.88	10.2	31.8	48.2	20
0.0018 – 144 hours	60.3±2.3	25.2±2.0	14.3±0.2	22±2.2	56.8±0.4	21.1±2.5

*The efficiency of transfection measured by GFP expression was 3.9±0.1%

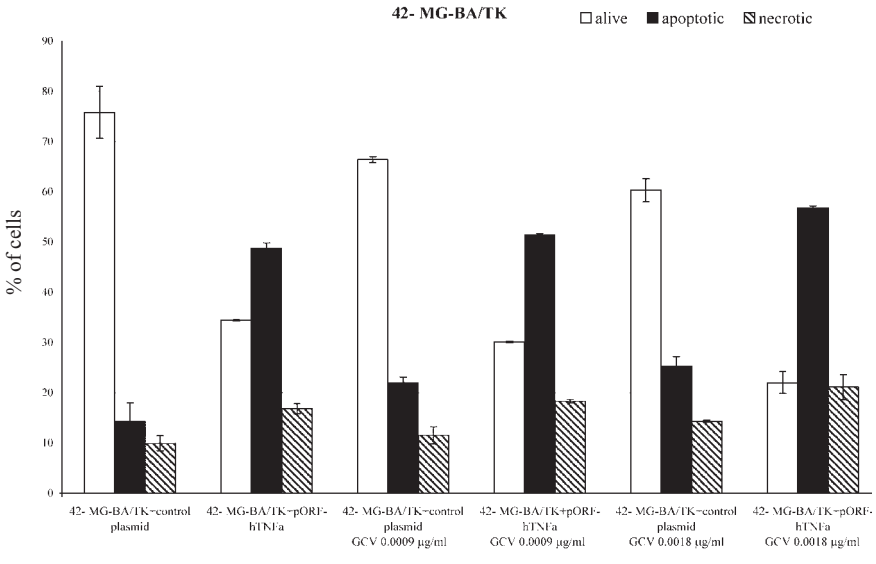

Figure 9. Induction of apoptosis by treatment of 42-MG-BA/TK cells transfected with control plasmid pORF-minus-hTNFα, and 42-MG-BA/TK cells transfected with pORF-hTNFα with GCV. Double annexin-V-FITC and PI staining of *HSVtk* transduced and 5-days GCV treated 42-MG-BA cells revealed the increased apoptotic portion of cells, which was enlarged after hTNFα expression.

more efficient leading to greater proportion of tumor cells dying by apoptosis. Based on the results obtained the construction of novel retroviral vector containing both suicide gene and *hTNFα* gene might lead to improvement in cancer gene therapy.

We wish to thank Mrs. M. DUBROVCAKOVA for technical assistance.

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