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Suicide Gene Therapy by Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK)

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1. Introduction

Suicide gene therapy for cancer treatment proposed by Moolten [1] started more than 25 years ago and has gained momentum with little variations in the original technology. At the current statistics, worldwide 1550 clinical trials of gene therapy have been reported (<http://www.wiley.co.uk/genmed/clinical>) among them 7% using suicide gene therapy. HSV-TK is the most well characterized suicide gene used for cancer therapy and in other diseases without inducing significant systemic toxicity [2] [3]. Chemotherapeutic drugs used for cancer therapy are problematic because they do not discriminate in their mode of action. Currently available drugs in the market are not cancer specific so functional concentration level in tumors cannot be reached without off-target toxicity level. This is specifically true for solid tumors where vascularization is poor and necrotic at the center of the tumor due to low oxygen and nutrient supply [4][5]. However, when the drug is activated by locally enzymatic reactions in a timely fashion, the metabolite is toxic for the tissue. The most widely used suicide genes are Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK), and Cytosine Deaminase (CD) from the virus Herpes simplex or the bacterium *Escherichia coli* respectively [6][7].

Prior research has focused on the mechanism of initiation and development of tumors. It starts with the mutation of 1 or several genes, then gradually attains more genetic mutation and genomic instability during the evolution of tumor/cancer. Almost all the genes fall in 2 categories - oncogenes (derived from proto-oncogene) and tumor suppressor genes. There are many ways to treat various tumors ranging from benign to metastatic. Among them surgery followed by chemotherapeutic drug treatments is the widely used method of choice that damages the DNA and renders the cells apoptotic. Additionally, tumors can be cured by more subtle ways by restoring the function of tumor suppressor genes or disabling oncogenes, to prime the immune cells to act and down-regulate angiogenesis and metastatic activities. Solid tumors can be treated by intra-tumor delivery of suicide genes. The protein product of these genes catalyzes the formation of highly toxic metabolites following the application of some lesser toxic prodrugs. This leads to apoptosis or programmed cell death of the treated cells [8]. The apoptotic cells invite immune cells that further clear the tumor zone by phagocytosis.

Suicide gene therapy is also known as Gene Directed Enzyme/Prodrug therapy (GDEPT) or as Gene Prodrug Activation Therapy (GPAT). GDEPT can either take the form of CBT (cell

based therapy) where nearby cells of the tumor are modified to express suicide gene or the tumor itself expresses the suicide gene therapy. GDEPT can also be used for viral vectors which itself carries the suicide gene. GDEPT (Molecular chemotherapy) has advantages over classical therapy. As only tumor cells possess the enzyme that converts prodrug to active metabolites, it increases the toxicity level several fold inside the tumor whereas the vast majority of the host cells are unaffected. By using tumor specific regulatory elements (promoter) drugs can be activated only in tumor cells [9][10] has used the technique where the drug is only preferentially activated in the hypoxic regions of the tumor. By harnessing the bystander effects, more destruction of tumor cells have been rendered.

2. Properties of suicide gene

To be considered as a suicide gene, the product enzyme should be either absent or present in low concentrations (low expression) in the host. It should have a high catalytic activity, so that tumor cells can convert this prodrug even in low substrate concentration (high K_{CAT} , low K_m). To be considered as an ideal therapeutic agent, it should have/possess the following criteria. 1) The drug should be non-toxic or minimally toxic prior to activation and highly toxic after enzymatic activation. 2) The prodrug should be able to effectively penetrate the tumor, distributed and taken up by individual cells. 3) It should have a high affinity for the transduced suicide gene and low affinity for cellular enzyme. The HSV-TK enzyme has 1000 times more affinity for the substrate GCV than the host cell TK [11]. 4) The metabolite should have a half-life that extends long enough to kill the tumor so that drug is not lost before reaching its concentration.

3. Types of suicide genes for therapy

There are several suicide gene therapies. Among them HSV1-TK and cytosine deaminase are important. Cytidine Deaminase: Cytosine Deaminase (CD) is an enzyme found in some bacteria and fungi that deaminates cytosine to uracil [12]. It can also convert the nontoxic 5-fluorocytosine (5-FC) into the toxic compound 5-fluorouracil (5-FU) [13]. Mammalian cells do not contain CD. This property allows the drug to be used for suicide gene therapy especially for the treatment of cancer. The sensitivity of tumor to CD drug depends on dose, duration etc. This review will mainly focus on HSV-TK gene therapy.

HSV-TK: TK converts GCV into a toxic metabolite that kills cells widely used for cancer therapy. HSV1-TK can also phosphorylate various nucleoside analogs of GCV such as acyclovir, ganciclovir, and penciclovir.

4. Mode of action of HSV-TK

HSV-1 encodes about 70 genes some are immediate early genes. TK is one of the immediate early (IE) genes that give rise to a 376 amino acid long protein. IE genes can be defined as genes that show rapid and transient expression in the absence of *de novo* protein synthesis; some viruses possess IE genes. HSV1 virus is neurotropic (preferentially attacks nerve cells to avoid immune cells) and TK is necessary when the virus goes into the lytic cycle from a dormant stage in the neural cells. During TK suicide gene therapy, GCV is injected systemically way and upon reaching the tumor area, the drug is monophosphorylated by HSV-TK, and further phosphorylation is done by the host cell kinase. This triphosphate

form of GCV (deoxythymidine triphosphate) is an analog of purine which inhibits DNA polymerase and is the most toxic. Cancer cells are actively proliferating and synthesizing DNA; the purine analogs GCV triphosphate competes with triphosphate substrate for DNA polymerase and are incorporated in the nascent DNA. This results the DNA polymerase enzyme stall and termination of nuclear and mitochondrial DNA synthesis initiating the cascades of apoptosis paths. [14][1]. The mechanism for cell death with HSV-TK is not completely known. Apoptosis induction or the sensitization of CD95- L, TNF, and TNF-related apoptosis-inducing ligands may contribute to cell death [15]. Yang *et al.* suggested that apoptosis occurred as a result of GCV-induced cell cycle arrests rather than direct chemical effects [16]. Depletion of the T-cells had no effect on the response [17]. The bystander effect causes local inflammation and the apoptotic cells invites dendritic cells and immune effectors (immune response) and further clears the tumor [18][19].

5. Bystander effect mediated by gap junctions

The bystander effect can amplify the effect of toxic drugs several fold. The toxic form of the drug should have diffusible property so that it can kill the non-transduced tumor cells through the bystander effect; in case of absence of diffusion, it should be taken up by surrounding cells by active transport. The action/effect of drug should also be cell cycle independent. Although cancer cells are highly proliferating, at a particular given time only a fraction of cells are dividing. In these cases drug distribution by diffusion is helpful. Moreover, in case of solid tumors only 10% of the cells can be transduced; in that case tumor ablation is mainly dependent on the bystander effect. Phospho-GCV is about a 500 daltons molecule. Such a small molecule should spread to surrounding cells by diffusion. But phospho-GCV is not dissolved in the cell lipid membrane. So, they spread to neighboring cells by gap junction that mainly consists of connexin family of proteins among them connexin-43 is the most studied. Gap junctions are a narrow connecting channel (2-3 nm diameter) between cells that facilitates the exchange of small molecules less than 1.5 kd in size. The gap is bridged by connexins, a family of 21 proteins. Gap junction exchanges small metabolites, second messengers and electric signals through a procedure called Gap Junctional Intercellular Communication (GJIC) [20]. Gap junction allows the ablation of the entire tumor although all cells in the tumor do not contain the suicide gene. This phenomenon is known as bystander effect and has boosted/amplified the toxic effect. Many kinds of cells express gap junction and are connected with neighboring cells. Some of the brain tumors (gliomas) do not express gap junctions or downregulate gap junctions [21]. Several studies have been done to deliver connexin-43 with the suicide gene so that the bystander effect through diffusion of active drug can take place. This was attempted by the pharmacological administration of cAMP analogs, hydroxyurea etc. [22]. Various reports of using connexin-26 and connexin-43 for the augmentation of the bystander effect have been published [23][9]. Solid tumors from both humans and rodents express lower amount of connexin and gap junction [24]. Established cell lines derived from tumors also downregulate expression of connexin and sometimes lack gap junction. Fusion proteins consisting of HSVtk and 11 amino acids from HIV-1 TAT protein have been demonstrated to provide gap-junction independent intercellular trafficking [25].

6. Variations of original HSV-TK approach

HSV1-TK suicide gene therapy is used for glioma [26], prostate cancer [27], leukemia [28] and lymphoma. Potency of original HSV-TK has been improved by the application of

various strategies. An approach was made to deliver fusion protein of TK and viral tegument protein VP22 (to increase the bystander effect) [29] and by the use of fusion protein of HIV TAT and TK (that is more stable than wild type TK) [30]. Cerepro (sitimagene ceradenovec) is a recombinant adenoviral vector consisting of HSV-TK and replication deficient (where E1 and part of E3 genes have been deleted). After the brain surgery, this vector was injected immediately. Upon intra-peritoneal administration of GCV, the volume of the tumor was reduced [31]. It is found that GCV is a substrate for ABCG2 (ATP-binding cassette sub-family G member 2, also known as the breast cancer resistance protein) and glioblastoma side population can pump out small molecule drugs like GCV rendering it resistant to therapy whereas non-side populations are susceptible [32]. Due to toxicity of the surrounding healthy tissue especially liver parenchyma cells in liver tumor, the use of TK has limited its clinical usefulness [33]. This problem has been improved by injecting the vector inside the tumor or using engineered HSV-TK under tumor specific promoter like α -fetoprotein. [34]. Ad.TK was injected intratumorally in hepatocellular carcinoma (HCC) and it did not show any toxicity in normal liver tissue and the therapy was well tolerated. Ad.TK can be safely used in HCC patient upto 2×10^{12} viral Particles/patient [35]. The HSV-TK gene has been successfully transferred into hepatoma cells (BEL-7402), and the growth potential of these cells was significantly inhibited by the application of GCV [36]. The bystander effect was further boosted by a combination therapy of co-expression of TK and E-cadherin genes in adenoviral vector. E-cadherin expression modulates the gap junction by connexin expression. It is assumed that E-cadherin expression facilitates connexin transport to the plasma membrane thus connexin stabilized and minimize connexin internalization and degradation by lysosomes and proteasome mediated degradation [37]. The role of E-cadherin in suicide gene therapy is established in an *in vivo* model of pancreatic ductal adenocarcinoma. This way it was possible to increase the bystander effect by treating the tumor with HSV-TK+E-cad [38]. Additionally, the increase of E-cad expression. also down regulates bcl-2 (an anti-apoptotic gene) rendering cell death. Non-human primate marmoset is used as a transgenic model for preclinical studies. HSV-TK knock-in marmoset stem cell line (cmES) was established using RMCE (Cre recombinase-mediated cassette exchange). From the cmES cell line-generated tumor cells were effectively destroyed by GCV treatment. Thus, HSV-TK and GCV treatment may ensure safety of stem cell therapy [39]. In NSCLC (non-small cell lung cancer), human telomerase reverse transcriptase hTERT is up-regulated. Thus hTERT promoter controlled E1A (Ad.hTERT-E1A-TK /GCV) gene expression in NSCLC, efficiently killed different types of tumor cells and could be used a safe and potent therapy for NSCLC [40]. One group has used piggyBack vector for HSV-TK delivery and GCV treatment for gene therapy of cervical cancer [41]. Co-transfection of insulin like growth factor-I (IGF-I) and HSVTK by liposome promotes wound healing and minimizes the scar formation [42]. Adenoviral vectors containing HSVTK were transfected into T47D human breast cancer cells [43]. When grown in nude mice, administration of GCV markedly demonstrated regression of tumors over control animals [44]. Fong *et al.* demonstrated that ablation of CT26 tumor cells *in situ* was achieved by directly injecting high-titer HSV-TK retroviral vector preparations into the site of tumor cell inoculation followed by intra-peritoneal delivery of GCV [45]. Chondrosarcoma cells implanted into nude mice were injected with HSV-TK. After 4 weeks, the growth of tumors was significantly prevented [46]. Suicide gene therapy can be used as

a tool for molecular biology research. Evans G *et al.* have used HSV-TK suicide gene therapy to shut down Nerve Growth Factor (NGF) when its intended job is done i.e. the bridging of nerve gaps have been achieved [47]. Suicide gene was used as a molecular off switch for growth factor expression in a tissue engineered construct after the successful healing of the defect, both morphologically and functionally. NGF-producing HEK-293 called as hNGF-EcR-293 cells were genetically modified to incorporate HSV-TK gene as a suicide gene for cell kill upon treatment with GCV. The combination of the inducible NGF expression system with the HSV-TK system offers regulation of time and presumed dose-dependent NGF expression at the site of the lesion with a subsequent elimination of genetically engineered cells within the conduit.

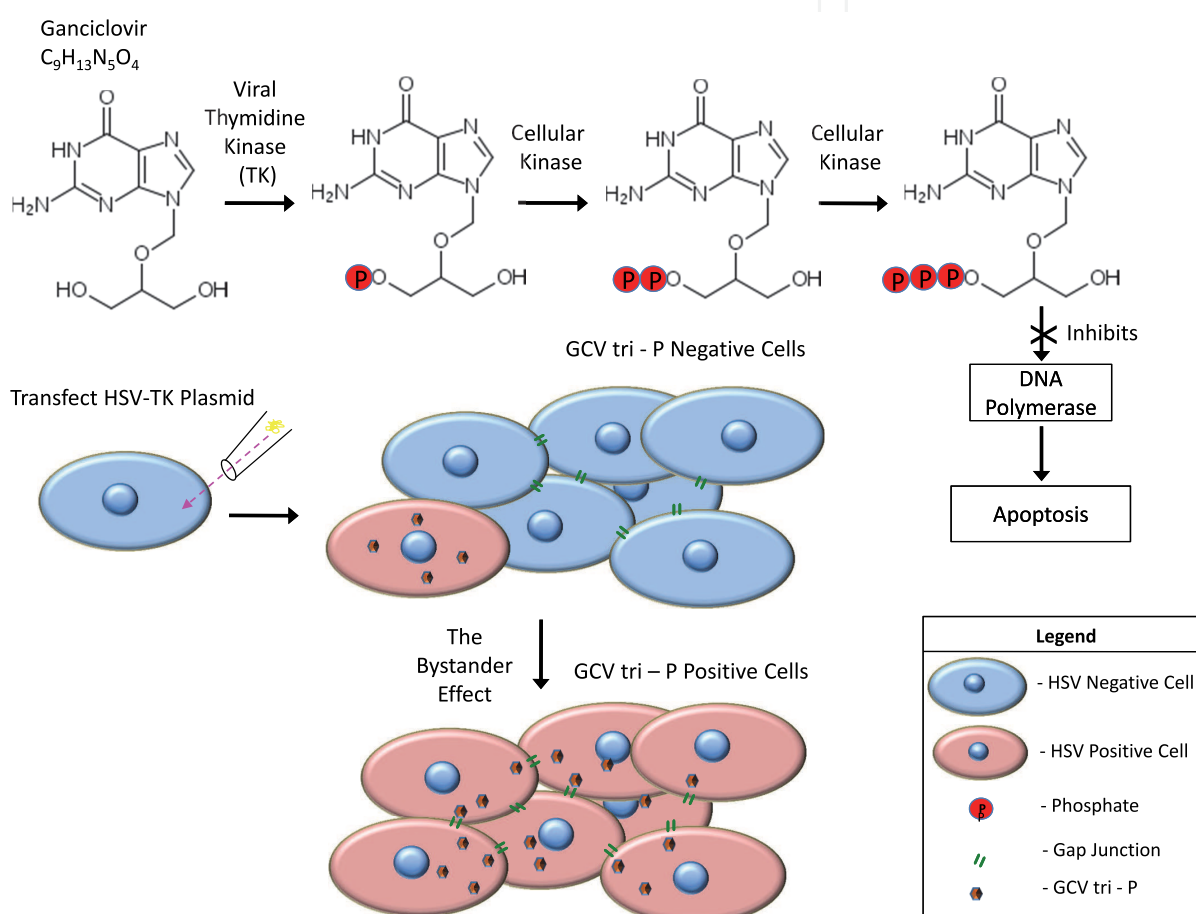


Fig. 1. Conversion of Ganciclovir by HSV-TK and cellular kinase to Ganciclovir triphosphate. The drug GCV is monophosphorylated by HSV-TK, and further phosphorylation is done by the host cell kinase. This triphosphate form of GCV (deoxythymidine triphosphate) is an analog of purine which inhibits DNA polymerase and is the most toxic. The bystander effect is mediated by the intercellular gap junctions present in many kinds of tissues and tumors.

One particular problem with the gene therapy is that retroviral vectors integrate in the genome randomly consequently it may potentially activate a proto-oncogene or silent a tumor suppressor gene rendering the cell to tumorigenesis. Insertion induced mutagenesis is a rare event at a frequency between 10^{-6} and 10^{-8} per insertion event [48]. Adenine

Deaminase (ADA) and Severe Combined Immuno-Deficiency (SCID) are genetic (inherited) disorders where the babies are born with immune deficiency and are vulnerable to common infections that others can resist in their everyday life. Several gene therapy approaches were used to deliver the corrected version of the gene in SCID patients by retroviral delivery. Patients regained immunity within 2-5 months but surprisingly several patients died due to cancer. Upon investigation, it was found that the virus has a preponderance to integrate in the vicinity of the human T-cell oncogene, LMO2 [49]. This probably due to retroviral transgene that has cotransforming role in leukemogenesis by giving advantages of increased cell proliferation or decreased apoptosis. As a cautionary measure, further trial of gene therapy was stopped. Delivery of HSV-TK fused with the therapeutic gene of interest into the therapeutic vector can be used for gene therapy. When the therapeutic gene has done its intended job, the cells harboring the gene and HSV-TK can be selectively eliminated by the administration of GCV. Thus killing the cells after gene therapy can be protective before the oncogene can do any harm. The GCV system has also been utilized in the non-oncologic setting. *In vivo* transduction with HSV-TK adenoviral vector followed by GCV treatment significantly inhibited the development of posterior capsule opacification from hyperplasia of the lens epithelium in the rabbit. Further, HSV-TK plasmid DNA has been injected into the joint space of rabbits with antigen-induced arthritis and when treated with GCV, results demonstrated a reduction in joint swelling in the HSV-TK-transduced knees [50]. Barbier *et al.* in an open and single-arm study on 48 patients, demonstrated that intracerebral injection of HSV-TK carrying cells into glioblastoma multiforme (GBM) did not result in any adverse effects [51]. HSV-TK has also been injected into the white matter of the right frontal lobe in two rhesus monkeys, with no clinical symptoms observed [52]. Murata *et al.* have demonstrated that like the Muristerone A-inducible system, the HSV-TK suicide gene allows for dose- and time-dependent regulation of cell death upon the application of GCV [53].

HSV-TK was modified to be able to migrate to neighboring cells and expand the expression of TK positive cells. A secreting form of HSV-TK was constructed by adding I κ gkappa leader peptide in the TK gene. An endoplasmic reticulum export signal was added to further increase the secretion. This resulted in the 70% of total protein secreted. However, enzyme activity of the secreted protein was decreased. This may present a hurdle for the development of a transmitted form of TK [54]. Microbubble destruction by ultrasound increases the efficiency of HSV-TK transfection [55]. HCC induces intrahepatic metastatic growth which is difficult to treat and prognosis is poor. A novel approach to introduce chemokine ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1) and HSV-TK together increases the efficiency of gene therapy. CCL2/MCP-1 attracts T helper 1-polarized antitumor activity without inducing tumor angiogenesis [56]. By using an IRES sequence, a plasmid was constructed with 2 suicide genes driven by PSMA promoter. The genes are FCY1 and HSV-TK for metabolizing the drug. Introducing the combination of 5-FC and GCV drugs inhibited the growth of prostate tumor compared to each drug individually. In a xenograft mouse model, retarded tumor growth was also observed. This suggests that the combination of multiple suicide genes may be more effective in prostate tumor [57]. Adenovirus mediated HSV-TK is a promising adjuvant therapy for patient's having high grade glioma. This is a choice as surgery for malignant glioma is difficult due to its location and non-metastatic nature of glioma. The HSV-TK attacks the dividing tumor cells without harming healthy neurons which are non-dividing [58]. Pancreatic tumor was treated with

mesenchymal stem cells (MSC) containing a CCL5 promoter. Homing of MSC cells into primary pancreatic tumor stroma and activation of the CCL5 promoter takes place. About one week later after stem cell treatment tumor size reduced to 50% and metastasis reduced significantly [59]. Use of undifferentiated embryonic stem (ES) cells may form teratomas thus limiting the use of stem cells in clinical setting. However, HCV-TK with Oct4 promoter construct was injected to ES cells. Upon treatment with GCV, undifferentiated cells die but differentiated cells are free from harm [60]. Combination gene therapy using multidrug resistance (MDR1) shRNA and HPV-TK [61], targeting angiogenesis of hepatocellular carcinoma with GCV treatment has been done [62]. Transfection of wild type p53 makes C6 glioma cells more susceptible to GCV treatment [63]. HIV-1 transactivator protein transduction domain (TAT PTD) can penetrate the cell. Cytotoxicity of GCV was enhanced by the fusion of HSV-TK and TAT PTD [64]. Fusion of mutant HSV-TK (with improved GCV activation) and guanylate kinase enhances prodrug sensitivity [65].

7. Disadvantage or drawback

GCV has toxic side effects especially on bone marrow cells. So, it is administered in lower concentrations [66] and has been used in animal model studies. The disadvantage of using GCV is that although GCV is readily diffusible, its metabolite triphosphate is membrane insoluble and can't diffuse to surrounding cells. However, the bystander effect of the close proximity/ neighboring cells happen as triphosphate is transported to nearby cells through the gap junctions.

8. Conclusion

Suicide gene therapy is a method of choice to ablate cells in many diseases including cancer. But like other techniques this method is not fully safe and efficacious. HSV-TK gene therapy is still evolving and the method has been tinkered to be applicable in various cell background and different goal. More works need to be done for future applications.

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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