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Tumor killing using the HSV-tk suicide gene

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Summary

The Herpes Simplex virus thymidine kinase gene (HSV-tk) has been widely used as a suicide gene in cancer gene therapy. Since the first demonstration of the ability of HSV-tk gene modified tumor cells to generate a bystander effect, a number of clinical trials have been initiated to treat human cancers. However, the mechanism of the HSV-tk mediated bystander tumor killing has remained controversial and is under intense investigation. The present report discusses the various mechanisms proposed by which the HSV-tk mediated bystander tumor killing occurs and highlights the importance of the host immune system in mediating the tumor killing. In addition, the present report also demonstrates that the initial tumor killing results in an inflammatory response leading to a cytokine cascade. This subsequently leads to an immune response resulting in an influx of macrophages and tumor infiltrating lymphocytes. Finally, strategies to augment the HSV-tk mediated bystander tumor killing by immunization are discussed and conclude with potential pitfalls of using HSV-tk/GCV system in a clinical setting.

I. Gene therapy

Gene therapy has been defined as the alteration of the genetic material of a cell with resultant benefit to a patient. Gene transfer has two broad categories : one in which a therapeutic gene is delivered to the cells with the aim of treating a disease; and second where a marker gene is delivered to label a cell type to determine the fate of a cell or the marker gene. Gene therapy is now becoming a rapidly developing therapeutic modality for experimental treatment of some cancers and diseases that have no alternative treatment (Anderson, 1992; Friedman and Roblin, 1972).

II. Suicide genes and suicide gene therapy

Definition of suicide gene: Suicide gene was originally developed as a safety measure to control the expression of a foreign gene introduced into a cell such that the gene modified cell can be eliminated if gene expression is no longer desired or if the gene modified cells become transformed. (Blaese, 1992).

During the course of developing the suicide genes, it was realized that if the suicide gene can be delivered directly to a tumor, they can be used for cancer therapy. This concept forms the basis for suicide gene therapy.

The most common strategy utilized in suicide gene therapy involves the delivery of a gene encoding an enzyme that will metabolize a nontoxic prodrug into a toxic metabolite, leading to killing of the cells expressing the gene. The activated prodrug interferes with the replication of the transfected cells, while not affecting the non transfected cells. Therefore, systemic toxicity is minimal making this approach attractive for tumor gene therapy or as a safety device in the use of live tumor cell vaccines. The two most commonly used suicide genes, which have progressed into clinical trials, are the herpes simplex virus thymidine kinase (HSV-tk) gene coupled with the pro-drug ganciclovir (GCV) and the cytosine deaminase (CD) gene coupled with the pro-drug 5' fluorouracil (5-FU) (Freeman et al., 1992a; Mullen et al., 1992; Huber et al., 1994). Other candidate suicide genes which are being tested include the xanthine guanine phosphoribosyl transferase (XGPRT) and purine nucleoside phosphorylase (Besnard et al., 1987, Mroz and Moolten., 1993).

III. HSV/tk GCV system and the bystander effect

The herpes simplex virus thymidine kinase (HSV-tk) gene is the most commonly used suicide gene. Initially, Moolten et al., (1990) demonstrated that tumors cells expressing the suicide gene (HSV-tk) can be specifically killed both in vitro and in vivo when exposed to the anti-viral drug, ganciclovir (GCV). The HSV-tk gene specifically monophosphorylates the guanosine analogue ganciclovir (GCV) which is subsequently converted into the toxic GCV-triphosphate form by endogenous mammalian kinases. The GCV-triphosphate is incorporated into replicating DNA by cellular DNA polymerase, thereby arresting DNA replication and causing cell death (Elion, 1980). The HSV-tk enzyme is almost 1000 fold more efficient at monophosphorylating GCV than the cellular thymidine kinase (Elion et al., 1977). Therefore, GCV is highly toxic to cells that express HSV-tk but are minimally toxic to unmodified or uninfected cells at therapeutic concentrations of the drug (1-10mmol/L). However, neutropenia can be a clinical manifestation as result of GCV (Shepp et al., 1985; Elion, 1980; Freeman et al., 1996). The phosphorylation of GCV curtails its movement across cell membrane resulting in a longer half life (t1/2=18-24 hrs) within the cells than unmodified GCV (Elion, 1980). The increased half life of GCV is an important feature in the anti-tumor effects of HSV-tk gene modified tumors.

Based on the evidence that most cancers are clonal in origin, and that HSV-tk gene modified tumor cells are sensitive to GCV, initial strategy was to generate a mosaicism within an individual such that cells become HSV-tk positive randomly (Moolten et al., 1986; Moolten et al., 1990a). Any tumor arising later from one of the HSV-tk sensitized cells, then all the tumor cells will carry the sensitivity gene as a clonal property and thereby can be treated with GCV to eliminate the tumor (Moolten et al., 1990b). Additional drug sensitivities can be achieved by using a combination of suicide genes (e.g.: CD and XGPRT) such that a complete mosaicism can be obtained. In such a situation, cells expressing three different kinds of suicide genes would exist within an organ. If a cancer developed later from a cell carrying any one of these genes, then those cells can be selectively eliminated by using the appropriate drug treatment. Thus, the normal nonmalignant cells will be spared with very minimal damage and thereby can repopulate.

Although the mosaic theory for cancer therapy using suicide genes is an attractive approach, due to current limitations in the available technology it may not be immediately applicable in the clinic. The various difficulties currently faced include (**i**) inefficient gene transfer into cells of an organ in particular when retroviral vectors are used as a result of which only a small portion of an organ can be modified (**ii**) transient gene expression when adenoviral vectors are used as a result of which, gene expression is lost in a rapidly dividing cell population (e.g.; malignant growth) (**iii**) silencing of the gene (e.g.; methylation) resulting in loss of expression of the recombinant protein (**iv**) incomplete tumor killing. Thus modifications of the existing approach is required before suicide gene therapy can be applied as a prophylaxis for cancer.

Since it is difficult to genetically modify all tumor cells within an individual, killing of unmodified tumor cells needs to occur in order for this approach to be therapeutically effective. Using the HSV-tk/GCV system, Freeman and colleagues (1992a; 1992b; 1993) demonstrated that HSV-tk gene-modified tumor cells are toxic to nearby unmodified tumor cells when the mixed tumor population is exposed to GCV. This phenomenon where untransduced tumor cells, not expressing the HSV-tk enzyme, are killed has been termed the "bystander effect". The effectiveness of the "bystander effect" to kill tumor cells has been shown both in vitro and in vivo and occurs even when only a fraction (10%) of the tumor mass contains the HSV-tk gene-modified tumor cells (Freeman et al., 1993). In addition the bystander effect has been demonstrated when syngeneic or xenogeneic HSV-tk gene modified tumor cells were used, indicating that irrespective of cell type, the gene modified cells need to be in close proximity to the unmodified tumor cells for the antitumor effect (Freeman et al, 1995a). Several other investigators have subsequently demonstrated the occurrence of the bystander effect using different tumor cells lines expressing the HSV-tk gene (Culver et al., 1992; Vile et al., 1993; Barba et al., 1993; Ram et al., 1993). The demonstration of the bystander effect has important implications in cancer therapy since it removes the burden of the need for delivery of the gene to 100% of the tumor cell population.

The use of HSV-tk/GCV system in the treatment of cancer offers several advantages : (i) rapidly replicating tumor cells are more susceptible to impairment of DNA synthesis (ii) chemotherapy resistant tumors can be made sensitive when genetically modified with the HSV-tk gene and (iii) HSV-tk/GCV-treated tumor cells have the ability to kill neighboring tumor cells through the bystander effect. Such a strategy has been tried to treat various experimental tumors (Culver et al., 1992; Ezzedine et al., 1991; Takamiya et al., 1992). After some encouraging results from experimental animal studies, many clinical trials have been approved worldwide (Freeman et al., 1995b; Clinical Protocols 1993; Clinical Protocols 1994a; 1994b). Although clinical protocols have been initiated, the precise mechanism of the bystander effect is unclear and is currently under intense investigation (Kolberg, 1994; Seachrist, 1994). Several hypothesis have been proposed for the mechanism of bystander effect which includes : apoptosis, endocytosis of toxic cell debris, blood vessel destruction and the involvement of the host immune system. In addition, reports from several groups indicate that the bystander killing varies depending upon the type of tumor cell used. Whatever the mechanism is, the generation of the bystander effect explains at least in part, the success of the delivery experiments in vivo that have successfully eradicated growing tumors despite the improbability of having delivered HSV-tk to every tumor cell. The observation and

results from our laboratory and others, studying the mechanism of the bystander effect, will be discussed in detail in the following sections.

IV. In vitro HSV-tk mediated bystander effect

Since the initial findings by Freeman et al., (1992a, 1992b, 1993) demonstrating the occurrence of a bystander effect, the mechanism of bystander tumor killing has been controversial and has been the subject of intensive investigation. Initial in vitro studies suggested that toxic metabolites of GCV from HSV-tk gene modified tumor cells contained in apoptotic vesicles were transferred to the adjacent unmodified tumor cells by phagocytosis (Freeman et al., 1993). This was based upon the observation that HSV-tk gene modified tumor cells when exposed to GCV undergo apoptotic cell death as evidenced by cytoplasmic shrinkage, chromatin condensation and nuclear DNA fragmentation. Additional in vitro studies demonstrated that the bystander tumor killing resulted from the transfer of toxic GCV metabolites through apoptotic vesicles to nearby unmodified tumor cells (Samejima et al., 1995; Colombo et al., 1995).

However, subsequent studies by Bi et al., (1993) using radiolabeled GCV demonstrated that the anti-cancer effect occurs in vitro by the transfer of toxic GCV metabolites from the dying HSV-tk tumor cells to the adjacent unmodified tumor cells through gap junctions. Similar results demonstrating the role of gap junctions in HSV-tk mediated bystander killing have been reported by other investigators (Fick et al., 1995; Elshami et al., 1996). Like other nucleotides, phosphorylated GCV cannot pass through the plasma membranes except when traversing to neighboring cells by gap junctions. Gap junctions are intercellular communicating channels that connect adjacent cells and which are in dynamic equilibrium exchanging ions and proteins between cells. These channels are permeable to molecules smaller than Mr 1000, such as cyclic AMP, calcium, and inositol triphosphate, but do not allow the transfer of proteins and nucleic acids. Gap junction channels are formed by proteins called connexins. The family of connexin proteins include at least 13 members in rodents. The role of connexins, in particular connexin 26 (Cx26) in gap junctional mediated bystander killing in vitro was demonstrated by Mesnil et al., (1996). More recently, connexin 46 (Cx 46), a tumor suppressor gene, has also been demonstrated to mediate the bystander tumor killing (Mesnil et al., 1997). Tumor cells when cotransfected with Cx23 or Cx46 along with HSV-tk gene showed enhanced bystander killing when exposed to GCV. In contrast, tumor cells transfected with HSV-tk alone showed decreased cell death while cells transfected with Cx23 or Cx46 alone showed no cell death upon exposure to GCV. Although gap junctions probably play a key role in the mechanism of bystander effect in vitro, their role in the in vivo bystander tumor killing has not been tested.

V. In vivo mechanism of bystander tumor killing

Although the mechanism of HSV-tk bystander tumor cell killing in vitro has been demonstrated to occur between cells in close proximity through gap junctions, the in vivo mechanism of tumor killing remains unresolved. This is partly due to the conflicting reports that have been generated using different tumor models. However, results are now emerging from several laboratories including ours suggesting that additional mechanism may be operational in vivo, namely the host immune system.

The observation that the host immune system participates in mediating the bystander effect in vivo stems from the initial findings demonstrating severely diminished or abrogated bystander tumor killing in animals that lacked an intact immune system, particularly T-cells (Freeman et al., 1992a, 1992b; Freeman et al., 1993; Freeman et al., 1994; Vile et al., 1994; Whartenby et al., 1995; Colombo et al., 1995; Ramesh et al., 1996a). Furthermore, HSV-tk gene-modified tumor cells and GCV can prolong animal survival when injected intraperitoneally (i.p.) into i.p. tumor bearing mice (Freeman et al., 1992; Freeman et al., 1993). In vivo autopsy results showed a rapid centralized hemorrhagic tumor necrosis which occurs within 24 hours after injection of HSV-tk gene modified tumor cells and GCV (**Figure 1**) suggesting that a more rapid mechanism of tumor killing was occurring (Freeman et al., 1994; Whartenby et al., 1995; Ramesh et al., 1996a). This is in contrast to the in vitro tumor cell death which is mediated by apoptosis that occurs over a period of 48-72 hours. The rapid occurrence of hemorrhagic tumor necrosis following the initial killing of the injected HSV-tk modified tumor cells after exposure to GCV indicated that soluble factors (cytokines and chemokines) which are capable of causing necrosis are released (Carswell et al., 1975; Schall and Bacon, 1994). This is due to the fact that tumor necrosis occurred inside, from the center of the tumor, rather than from the outside of the tumor on the periphery (**Figure 1**). The occurrence of tumor necrosis was also reported by Ram et al., (1994) using HSV-tk vector producer cells. However, the observed tumor necrosis was attributed to be due to the transfer of retroviral particles carrying the HSV-tk gene to the endothelial cells lining the tumors blood vessel, which were destroyed when exposed to GCV. Although, the HSV-tk delivery system used were different (Freeman et al., 1993; Ram et al., 1994), the occurrence of tumor necrosis was observed to be a common phenomenon in both the studies. Several other investigators have also subsequently documented the occurrence of tumor necrosis following HSV-tk/GCV treatment (Barba et al., 1994; Bovistias et al., 1994).

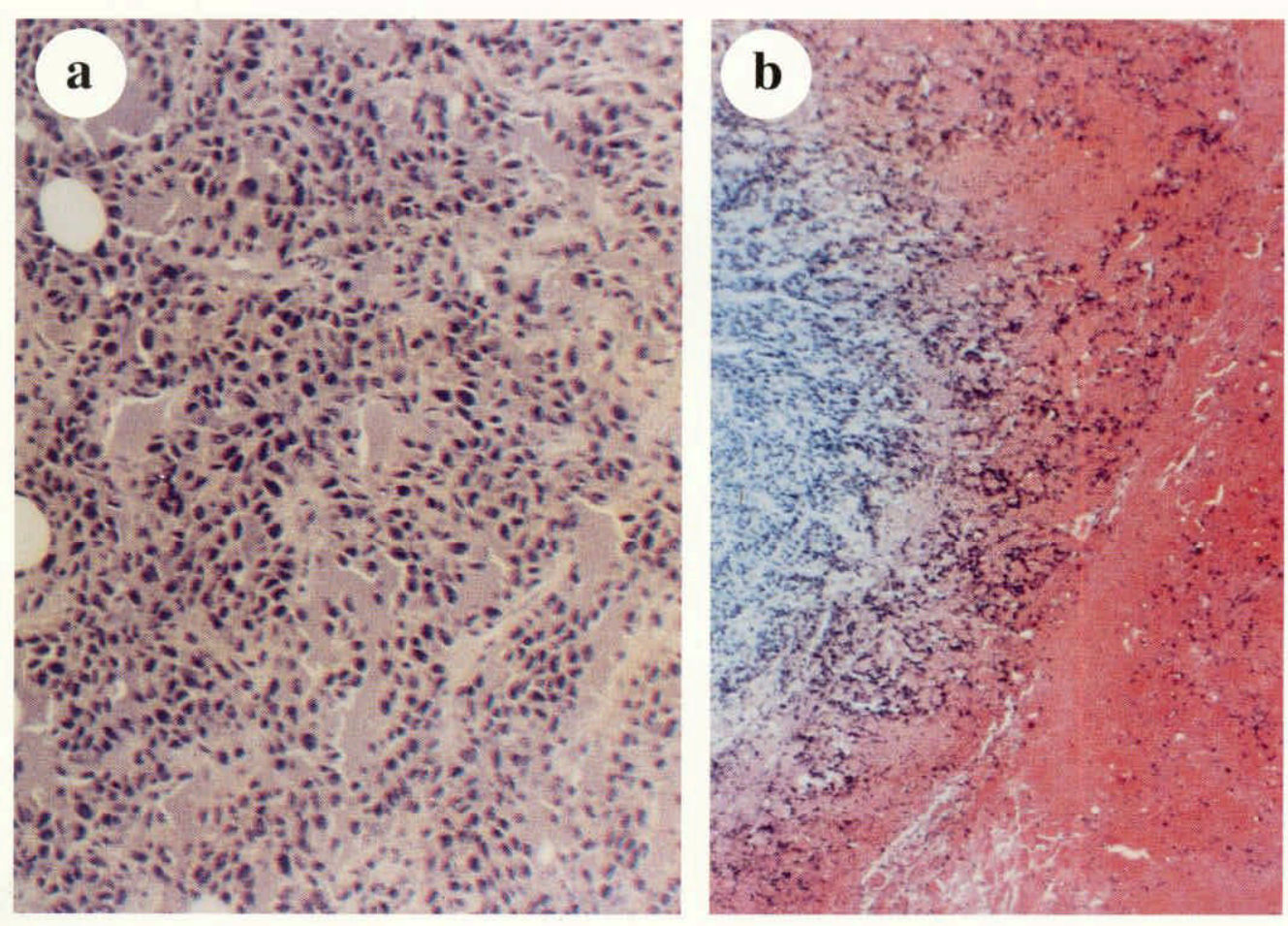

Figure 1: Hemorrhagic Tumor Necrosis. BALB/c mice with intraperitoneal murine tumors were injected with HSV-tk gene modified tumor cells with or without GCV. Tumors were harvested 24 hours later and examined microscopically by hematoxylin and eosin staining (H&E). **A**. Absence of necrosis in tumors not receiving GCV. **B**. Necrosis observed in tumors from animals receiving HSV-tk and GCV treatment.

Further evidence demonstrating that soluble factors are responsible for centralized tumor necrosis comes from the observation that when fluorescein labeled HSV-tk tumor cells are injected intraperitoneally into an i.p. tumor bearing animal, these cells preferentially homed to actively growing tumor in-situ (**Figure 2**). The argument for the role of soluble factors is based on the fact that if apoptotic cell death or gap junctional mediated bystander tumor killing was to occur following GCV treatment, then cell death would occur at the periphery of the tumor rather than on the inside (Shastri et al., unpublished data). Furthermore, apoptotic cell death leads to the upregulation of IL-1β converting enzyme (ICE) which causes IL-1 secretion (Hogquist et al., 1991).

Based on the known functions of some of the soluble factors such as tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) which can cause rapid necrosis, one can speculate that the observed tumor necrosis is caused by TNF-α and IL-1 (Carswell et al., 1975; Dinarello, 1996). To this extent, i.p. tumor bearing animals were treated with HSV-tk gene modified tumor cells and analyzed for cytokine production. Expression of TNF, IL-1 and IL-6 mRNA was observed within 24 hours following HSV-tk/GCV treatment which coincides with the observed centralized tumor necrosis (Freeman et al., 1994; Freeman et al., 1995a; Ramesh et al., 1996a). In addition, an increase in the message for TNFα was observed (Ramesh et al., 1996a).

Since TNFα and IL-1α are known potent activators of host anti-tumor cells such as macrophages, NK cells, and cytotoxic lymphocytes, and can serve to potentiate the proliferative response of T lymphocytes (Urban et al.,

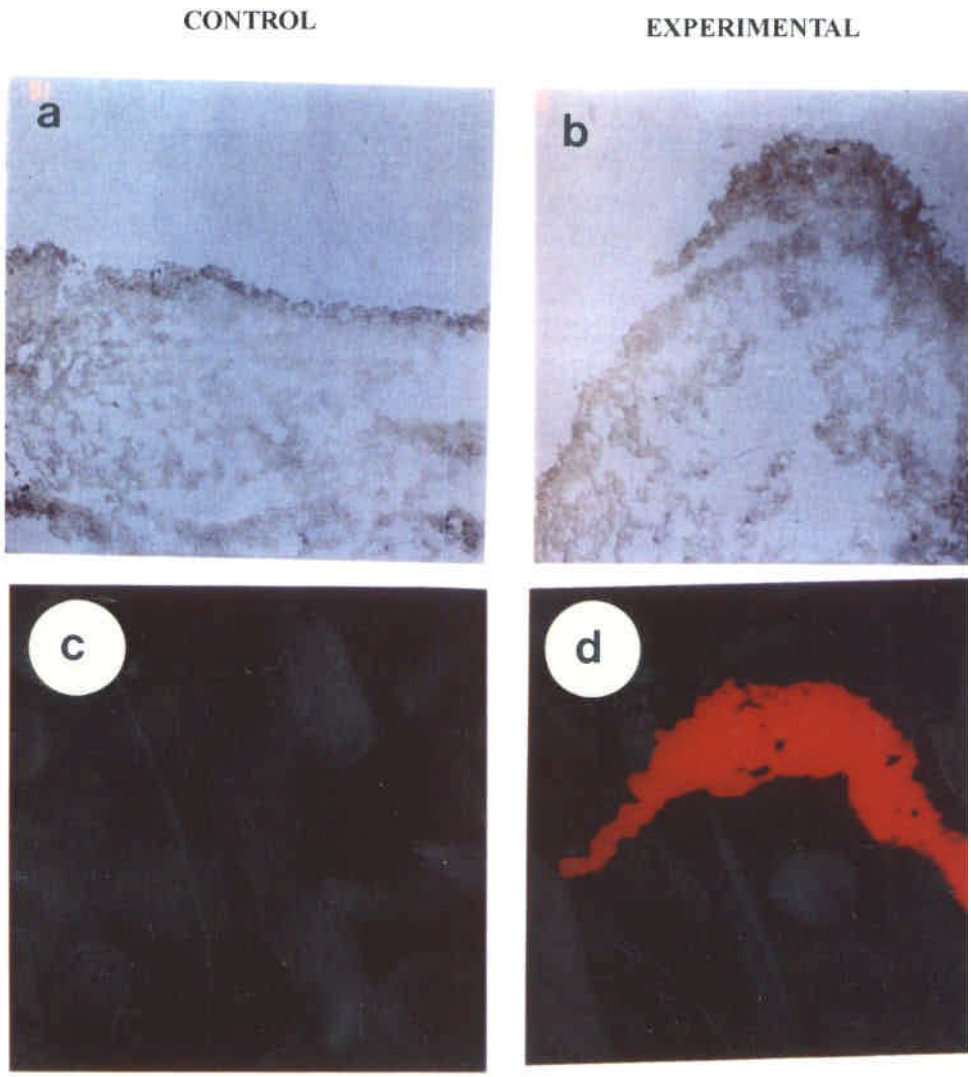

Figure 2: Homing of Fluorescein Labeled HSV-tk gene modified tumor cells. Fluorescein labeled (experimental) or unlabeled (control) HSV-tk gene modified tumor cells were injected intraperitoneally (i.p.) into i.p. tumor bearing mice and analyzed for their fate. The tumors were isolated 24 hours post injection and analyzed by light microscopy (**a** & **b**) and fluorescent microscopy (c & d). The HSV-tk tumor cells home onto actively growing in-situ tumor and adhere to the outer surface of the tumor as seen by the fluorescence in experimental animals (**d**). Unlabeled cells when injected do not fluoresce and were used as a control (**c**).

1986; Palladino et al., 1987; Nakano et al., 1989; Harada et al., 1994) immunohistochemical studies were performed on tumor tissues from control and experimental animals for infiltrating macrophages and T-cells. It was found that in the tumors from animals receiving HSV-tk and GCV treatment there was a significant increase in the macrophages and T cells as compared to the control animals (Ramesh et al., 1996a). Studies supporting these findings have been further documented by other investigators demonstrating infiltration of immune cells (macrophages, $CD4^+$, $CD8^+$) following HSV-tk/GCV treatment (Caruso et al., 1993; Barba et al., 1994; Vile et al., 1994; Pope et al., 1996; Gagandeep et al.; 1996). Increase in mononuclear and T cells within the tumor after injection of the HSV-tk gene modified tumor cells could be due in part to the TNFa induced hemorrhagic necrosis as well as increased up-regulation of adhesion molecules caused by inflammatory cytokines such as IL-1α. Similarly, the infiltration of effector T cells and macrophages into the tumor following HSV-tk/GCV treatment, may be a direct consequence of the induction of localized cytokine expression.

Although PCR can detect mRNA expression within the tumor it does not necessarily reflect protein expression, even though we can demonstrate an increase in TNF mRNA in the HSV-tk treated animals compared to the untreated

animals. Immunohistochemical studies showed that mononuclear cells infiltrating the tumor were expressing TNF and IL-1 suggesting that cytokines are generated in the process of development of an antitumor response (Freeman et al., 1994; Ramesh et al., 1996a). More recently, Vile et al., (1997a) using B16 melanoma tumor cells demonstrated the production of cytokines along with pronounced intratumoral infiltration of macrophages and lymphocytes following HSV-tk/GCV treatment in vivo. The ability to induce cytokine production in vivo has also been demonstrated for other agents such as the E. coli lipopolysaccharide (LPS). However, we found that LPS injections stimulated a different cytokine repertoire within the tumor than the HSV-tk tumor cells and did not prolong survival of tumor bearing mice indicating that in addition to cytokine production, the events that set the cascade appear to be critical (Freeman et al., 1996; Shastri et al., unpublished data). Based on these findings, we hypothesized that the HSV-tk gene-modified tumor cells alter the tumor's microenvironment from one that suppresses an anti-tumor immune response to a stimulatory one (Freeman et al., 1994; Ramesh et al., 1996a).

Failure of a tumor-specific T cell response in tumor bearing mice might in part result due to lack of tumor antigen expression or from the inability or inadequate expression of adhesion molecules (ICAM-1) and other cell surface molecules such as the costimulatory molecules (B7) by the tumor cells. Among the different accessory molecules expressed on antigen presenting cells (APC), cytokines can upregulate expression of co-stimulatory molecules like B7 and ICAM which have been suggested to play a major role in T cell activation (Freedman et al., 1987; Chang et al., 1994). B7 might be preferentially involved in stimulation of antigen primed T cells whereas ICAM-1 which is constitutively expressed on all APCs would be most efficient in co-stimulation of resting cells. The ability of pro-inflammatory cytokines (TNF, IL-1) to upregulate B7 (B7-1 and B7-2) expression has been demonstrated (Chang et al., 1995). Furthermore, B7 expression modulates the differentiation of T cells into Th1 or Th2 (Kuchroo et al., 1995).

Since HSV-tk/GCV treatment results in production of cytokines (TNF, IL-1, IFN-γ) in vivo, we investigated whether HSV-tk/GCV treatment could also elicit the expression of co-stimulatory molecules, B7-1, B7-2 and ICAM which are critical for the induction of anti-tumor immunity. Analysis for the expression of these cell surface immune regulatory molecules in-vivo after treatment of tumor bearing mice (i.p.) with HSV-tk gene-modified tumor cells and GCV demonstrated i.p. tumor bearing control mice do not express B7-1, while low levels of B7-2 and ICAM-1 are expressed (Freeman et al., 1995c; Ramesh et al., 1996b). Interestingly, it has been reported that low levels of B7-2 are expressed on the cell surface of naive leukocytes. Only upon activation does B7-1 become expressed. In mice with an i.p. tumor, inoculation of HSV-tk gene modified tumor cells with GCV led to up-regulation of B7-1, B7-2 and ICAM-1 thus indicating a state of activation within the tumor (Freeman et al., 1995c; Ramesh et al., 1996b). Furthermore, T-cells isolated from the spleen of tumor bearing mice treated with HSV-tk gene-modified tumor cells and GCV showed a proliferative response in-vitro to parental syngeneic tumor cells and released IL-2 which is often associated with an activated state. The proliferative response thus observed appeared to be specific since murine mastocytoma cells did not stimulate T-lymphocytic proliferation (Ramesh et al., 1996b). This type of response suggests that cells become activated after treatment with HSV-tk gene modified tumor cells and GCV with the generation of a tumor specific immune response in-vivo. The alteration in the tumor microenvironment following HSV-tk/GCV treatment has also been suggested by Vile et al., (1997a).

VI. Augmenting the HSV-tk mediated bystander killing by immunization

Based on the importance of the immune system in the generation of the "bystander effect", we examined whether enhancement of the immune system could augment the "bystander effect". This would be extremely important since it may not only be possible to treat local tumors but also metastatic tumors which are life threatening to the patient. Potential means for enhancement of the bystander tumor killing include (**i**) using biological response modifiers (BRM), such as cytokines to augment the immune/inflammatory response generated by the HSV-tk gene modified tumor cells and ganciclovir and (**ii**) immunization to a known tumor antigen before treatment with HSV-tk gene modified cells and ganciclovir.

Although active (tumor vaccination) and adoptive (TIL, LAK) immunotherapy has been extensively studied over the past decade, there has been only marginal clinical benefit to these approaches. One potential problem is that the peripheral blood "activated" immune effector cells generated by these approaches may become inactivated upon entering the immunosuppressive tumor environment. Thus, unless the tumor microenvironment can be altered, immunotherapeutic approaches may continue to have problems generating effective anti-tumor responses. The "activated" immune stimulatory environment developed by the inflammatory response to the HSV-tk gene-modified cells allows for the development of an immune response. But more importantly, it provides an environment for the efficient functioning of immune effector cells which exist within the host's peripheral blood. This latter issue relates to the enhanced anti-tumor response which we can demonstrate when combining immunization with HSV-tk gene-modified since immune effector cells in the peripheral blood generated by immunization can traffic to the tumor after treatment with HSV-tk gene-modified cells and GCV (Ramesh et al., 1997). However, the cell type used for immunization was found to be critical.

To further understand how this enhancement of the "bystander effect" occurs, we began to evaluate how the tumor environment is altered after immunization and treatment with HSV-tk gene-modified cells. Since we have previously demonstrated the occurrence of a centralized

hemorrhagic necrosis in vivo with release of soluble factors, in unimmunized mice treated with HSV-tk gene, cytokine mRNA expression was analyzed in intraperitoneal tumors by RT-PCR initially. We detected mRNA to all three cytokines (TNF, IL-1 and IL-6) in the tumors of mice within 24 hours in both the immunized untreated group and immunized treated group of mice, but could not detect the message in untreated mice. This observation is somewhat surprising since although it appears that the tumor environment can be altered by immunization, animal survival is unchanged by immunization alone.

More importantly, subsequent analysis for other cytokines demonstrated IL-2 expression only in mice immunized with syngeneic tumor and treated with HSV-tk gene-modified tumor cells and GCV. IL-2 was detected at 48 hours post injection of the HSV-tk gene-modified cells and GCV. None of the other cytokines tested (IL-10, GM-CSF, IL-4 and IFN-γ) were detectable in mice from any of the immunized groups, although results from our earlier studies demonstrate mice which were unimmunized and treated with HSV-tk gene-modified cells showed expression of GM-CSF and IFN-γ mRNA. The production of IL-2 only in mice receiving HSV-tk gene-modified tumor cells and GCV is intriguing since in our previous study using unimmunized mice receiving HSV-tk and GCV, IL-2 was not observed.

The demonstration of IL-2 mRNA after treatment may have significant implications in the enhancement of the "bystander effect" since it may activate T cells which in turn might be triggering and amplifying the antitumor effector response (Vieweg and Gilboa, 1995). Although, the subset of T lymphocytes (CD4/CD8) infiltrating the tumor was not characterized, T cells from immunized mice receiving HSV-tk gene-modified cells and GCV demonstrated an increased proliferation to syngeneic tumor than either mice immunized only or mice treated with HSV-tk/GCV only. In addition, an increase in the number of tumor infiltrating lymphocytes was observed. This is probably because of the hemorrhagic tumor necrosis which develops after treatment and the release of cytokines which allows the immune effector cells to enter the tumor and provides an immune stimulatory environment for them to function. Since the HSV-tk modified tumor cells and GCV can alter the tumor microenvironment to one that is immunostimulatory through the release of cytokines, as evidenced by increased expression of immune regulatory molecules such as ICAM-1 and B7, immune effector T cells already present secondary to immunization can function in the "activated" tumor microenvironment and kill the tumor. This situation, unlike that observed in unimmunized mice treated with HSV-tk tumor cells where only pro-inflammatory cytokines are produced, thus provides a potential new therapeutic cancer approach.

Attempts are currently being made to develop vectors for the delivery of HSV-tk gene along with other immunostimulatory genes that can be expressed at the same time as the cells are killed and can enhance the antitumor immune response. However, studies from various laboratories have met with varying degrees of success. For instance, when IL-2 secreting tumor cells were injected in conjunction with HSV-tk gene modified tumor cells, no enhanced bystander tumor killing was observed (Ram et al., 1994). Similar results were reported by Chen et al., (1995). However, combining HSV-tk gene modified tumor cells with IL-2 resulted in an increased long term tumor immunity in the surviving animals. In contrast, when interferon alpha (IFN-α) was combined with HSV-tk gene modified tumor cells, an enhanced bystander tumor killing was observed (Santodonato et al., 1996). Interleukin-12 (IL-12) which is a potent activator of T-cells is also being evaluated along with HSV-tk to enhance tumor cell elimination in vivo (Vile et al., 1997b).

Thus in-vivo therapy with cytokines capable of inducing either tumor cells or host immune cells to express molecules important in immunogenicity may be efficacious either independently or as an adjunctive therapy with HSV-tk.

VII. Proposed hypothesis

Based on the findings mentioned above, we would like to advance the following mechanism(s) to explain the bystander killing following HSV-tk/GCV treatment where the tumor microenvironment is altered from an immunosuppressed to an immunostimulated environment (**Figure 3**). Injection of HSV-tk gene modified tumor cells home to actively growing in-situ tumor through adhesion molecules. Primary killing of these HSV-tk tumor cells occurs with exposure to GCV resulting in an inflammatory response against the dying tumor cells which subsequently leads to an immune response. The inflammatory response generated by the dying HSV-tk gene modified tumor cells resembles the inflammatory response to microbial pathogens. This is partly because the HSV-tk gene modified cells die through apoptosis, which is facilitated by the transfer of toxic metabolites, releasing soluble factors such as TNF-α and IL-1α. This process then leads to hemorrhagic tumor necrosis with the simultaneous activation of leukocytes/lymphocytes (Th), by costimulatory signals (B7) and adhesion molecules (ICAM, VCAM) within the tumor resulting in the increased production of cytokines. The cytokines released within the tumor microenvironment may improve indirect tumor presentation by host cells and influence the type of immune mechanism(s) resulting in either a Th1 or Th2 like response. Furthermore, the chemotactic factors and cytokines produced regulate the influx of natural killer cells (NK), neutrophils, eosinophils and monocyte/ macrophages (Mac) into the site of inflammation or tumor deposit and thereby affect the tumor microenvironment. The initial inflammatory response generated is usually too weak to eliminate the entire tumor mass, allowing the tumor to grow to a size that is too large to be killed when anti-tumor immunity develops several weeks later. However, in immunized mice, the "activated" immune effector T cells ($CD4^+$, $CD8^+$) which are already present in the host's peripheral circulation possess strong anti-tumor activity which can function in the immune stimulatory tumor

environment generated by treatment with HSV-tk and GCV. Thus, this anti-tumor effect mediated by HSV-tk suicide gene therapy can be enhanced to be effective clinically.

VIII. Conclusions

Although, based on animal experimental studies a role for the host immune system in the bystander tumor killing in vivo has been demonstrated further studies are warranted. For example, neither the role of natural killer cells (NK) nor the role of macrophages in bystander tumor killing has been investigated. Till date, experimental studies have used T-cell deficient mice. However, these animals are neither deficient in NK cells nor in macrophages. Therefore, studies are required using animals which are completely lacking the immune system (NK and macrophages), for instance by using immune-deficient animals such as SCID or SCID-beige mice. In conclusion, suicide gene strategies using the HSV-tk/GCV system can be highly effective for treatment of local tumor growth or to switch off gene expression in adoptively transferred gene modified cells. However, these strategies can fail completely depending on the appropriate target cell. In particular, in a clinical setting, in vitro testing of the suicide effect may be useful to predict whether this approach has the potential to

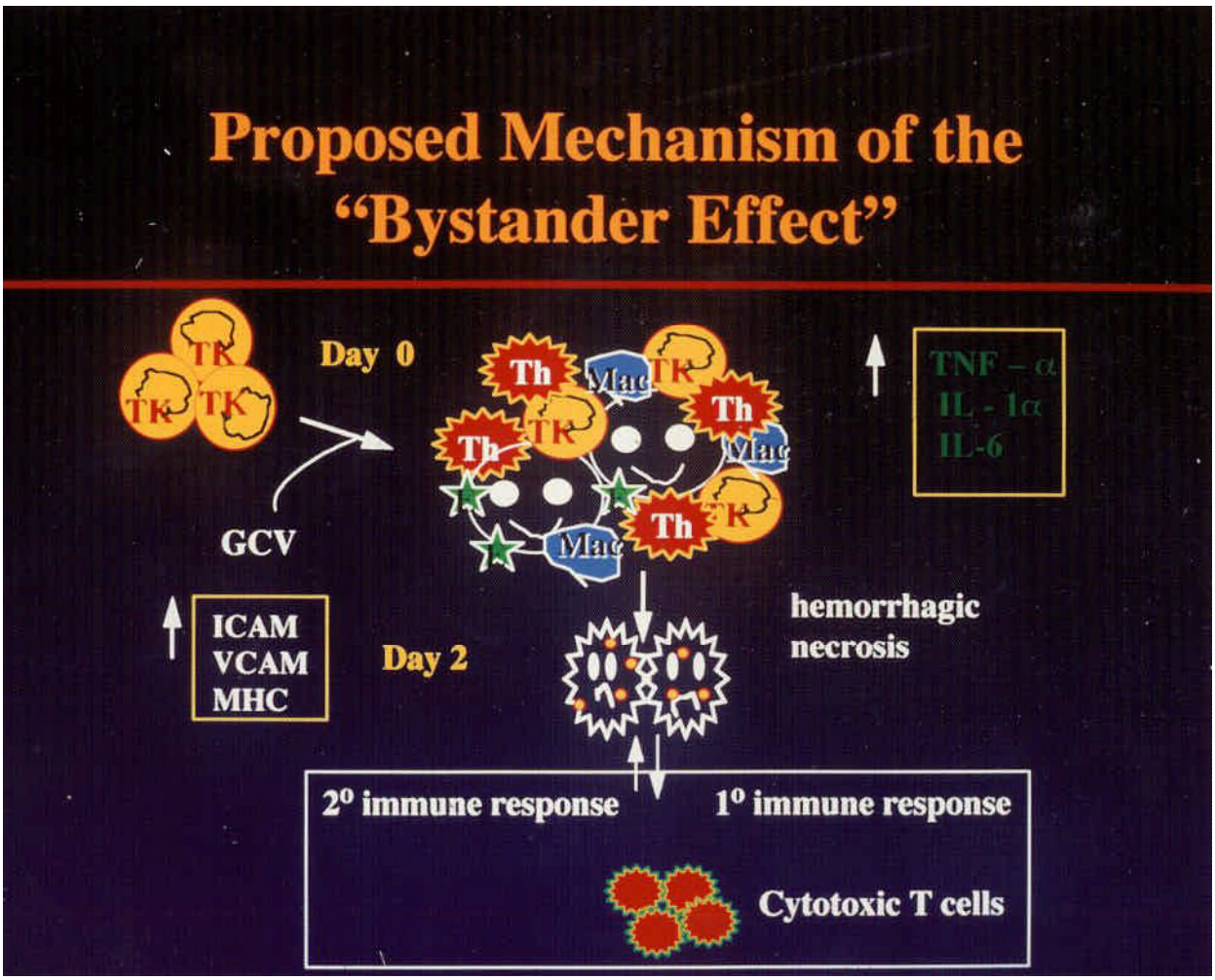

Figure 3: Proposed mechanism of the in vivo bystander effect. The injected HSV-tk gene modified tumor cells (TK) home to the actively growing in situ tumor. Treatment with GCV results in the killing of the HSV-tk gene modified tumor cells and the transfer of toxic metabolites to the adjacent bystander tumor cells resulting in hemorrhagic necrosis. The dying tumor cells (inflammatory response) release soluble factors (cytokines and chemokines) and shed tumor proteins. The resident macrophages (Mac) act as antigen presenting cells (APC's) resulting in the presentation of tumor antigens to the T-cells (Th). During this process, the cytokines (TNF, IL-1) upregulate the expression of costimulatory (B7) and adhesion molecules (ICAM, VCAM) on the lymphocytic infiltrates resulting in their activation. The activated lymphocytes produce more cytokines resulting in an influx of macrophages and T-cells (cytotoxic) which recognize the tumor antigens and kill the residual tumor (1^{o} immune response). Upon rechallenge the T-cells specifically recognize the tumor antigens (specific immunity) and kill any tumor cell present (2^{o} immune response) .

eliminate an individual tumor. Further understanding of the "bystander effect" will lead to improved uses for suicide gene therapy.

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