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Review

Suicide gene therapy with Herpes simplex virus thymidine kinase and ganciclovir is enhanced with connexins to improve gap junctions and bystander effects

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Summary. Connexins are proteins that form gap junctions between cells in various mammalian tissues. Because of their role in intercellular communication, connexins are important in the bystander cell death seen in Herpes simplex virus-thymidine kinase (HSV-TK) gene therapy for brain tumors. A selective review of connexin transduction/transfection studies with particular emphasis to central nervous system tumor cells is presented. In addition, specific references to studies with cell types that demonstrate low gap junction intercellular communication are presented. Data are included with the HT-29 colorectal tumor cell line to support the concept that enhancing gap junction protein expression in otherwise low gap junction communicating HT-29 cells increases bystander cell death and reduces tumor burden beyond what might be expected from HSV-TK and ganciclovir (GCV) treatment alone. Maximum *in vitro* bystander cell death was always produced when GCV treated co-cultures of TK-transduced and non-TK-transduced HT-29 cell lines were also transduced with connexin-43. When connexin was present in only one group of cells in the co-culture, there was more bystander cell death observed with connexin transduced into the non- TK-transduced cells, rather than the TK-transduced cells. The data presented reinforces conclusions made from earlier findings from cell line mixing experiments in which the non- TK-transduced cell population determined the level of bystander cell death (Burrows et al., 2002).

Key words: Glioma, Connexin, Gap junctions, Suicide gene therapy, Bystander effect

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Introduction

Brain tumors

The prognosis associated with malignant brain tumors is poor. While certain tumor types are associated with better survival rates than others, the estimated five-year survival rate for patients with malignant brain tumors is approximately 27%, and survival is known to decrease with age at diagnosis. For the most common malignancy, glioblastoma multiforme, the two year survival ranges from a high of about 30% for children and young adults, to a low of around 2% for patients over 65 (CBTRUS; 2000). In many cases the opportunity for treatment following initial diagnosis may be severely limited by the progression of the malignancy. During that time patients may suffer losses of function correlated with the location of the tumor in the brain and from the treatments that may themselves induce serious side effects. Even though surgical resection is common, it is generally cytoreductive due to the infiltrative property of the glioma cells and the diffuse demarcation of the tumor boundary. Radiation or chemotherapy is common as follow-up, but ultimately the tumors keep recurring until conventional treatment options are exhausted.

Gene therapy

There is an obvious need for new treatments for malignant brain tumors, and there are now various gene therapies that hold promise for extending lifespan (Sasaki and Plate, 1998; Weyerbrock and Oldfield, 1999; Bansal and Engelhard, 2000; Engelhard, 2000; Lam and Breakefield, 2001). In one form of gene therapy a new gene is introduced into a cancer cell for the purpose of sensitizing the cell for killing by later treatment with a

drug. The new gene encodes a protein that helps convert a prodrug to a toxic form when the malignant cell is exposed to the prodrug. The intention for the technique is to use gene transfer to selectively destroy tumor cells. Several gene delivery systems are available, notably, retroviral and adenoviral vectors (Sasaki and Plate, 1998; Bansal and Engelhard, 2000), and several gene/prodrug systems are known and have been applied, both to the solid tumors of glial malignancies as well as other cancers (Pope et al., 1997; Niculescu-Duvaz et al., 1998; Dilber and Gahrton, 2001).

The most common experimental and clinical model for gene therapy involves the transduction of the Herpes simplex thymidine kinase (HSV-TK) gene into tumor cells, followed by treatment with the antiviral drug ganciclovir (GCV) (Moolten, 1986; Culver et al., 1992; Oldfield et al., 1993; Ram et al., 1994, 1997). A diagrammatic representation of how the therapy is thought to proceed is given in Fig. 1. The experimental model typically involves an in vitro co-culture of groups of HSV-TK-transduced and non-TK-transduced tumor cells that can be assessed directly for the amount of cell death obtained after exposure to GCV. Alternatively, transduced cells can be prepared for injection into animals to study the course of in vivo tumor development or regression. The sequence of events leading to tumor cell death has been described in many reports (Mesnil et al., 1996; Pope et al., 1997; Boucher et al., 1998; McMasters et al., 1998; Rubsam et al., 1999). Briefly, the TK causes GCV to be metabolized to a monophosphorylated form, which is then converted to a toxic triphosphate form by other cellular kinases. The tumor cell dies when the toxic GCV metabolite is incorporated into replicating cell DNA, causing premature strand termination, replication failure and cell death. Because transduction of the tumor cell with the HSV-TK gene causes the cell to synthesize the enzyme which kills the cell, this type of prodrug activation is widely described as a "suicide" gene therapy in both the experimental and clinical literature (DiMeco et al., 1997; Rosolen et al., 1997; Suhr and Gage, 1999; Engelhard, 2000; Lail et al., 2000; Lam and Breakefield, 2001).

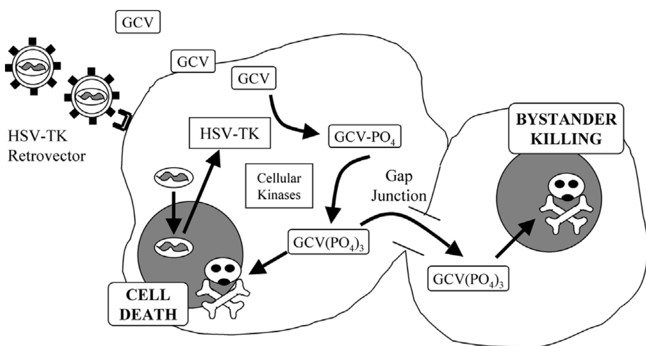

Fig. 1. Diagrammatic representation of how suicide gene therapy with HSV-TK and ganciclovir is thought to kill transduced and neighboring cells.

Although HSV-TK prodrug activation therapy is used to treat different types of cancer, it has advantages that make it particularly suited to malignant brain tumors. The therapy can be delivered directly into the brain at or near the tumor site, typically as an adjunct to surgical debulking. Particular to retroviral gene delivery systems, the *in situ* transduction targets cells that are actively replicating. In the brain where neural and connective tissues are quiescent, the brain tumor cells are largely selectively targeted as the ones into which the gene is most likely to incorporate.

In general, the relative safety of the treatment has been established in clinical trials, although some adverse effects of the HSV-TK/GCV treatment have been noted, such as confusion, seizure, headache, nausea, cerebral edema, and meningeal inflammation (Ram et al., 1997; Klatzmann et al., 1998; Shand et al., 1999; Packer et al., 2000; Trask et al., 2000). Therapeutic anti-tumor responses have been observed in certain patients (Ram et al., 1997; Klatzmann et al., 1998), but a satisfactory anti-tumor efficacy has not yet been found (Shand et al., 1999; Rainov, 2000; Singh et al., 2001). Successful treatment appears to have been limited by the low titer, first generation prototype vectors or vector-producing cell lines, and the low rate of in situ transduction, among other factors (Sasaki and Plate, 1998; Rainov, 2000). In this regard an active area of research has been aimed at increasing HSV-TK transfer into tumors and at development of methods to increase the amount or potency of HSV-TK incorporated into brain tissue (Howard et al., 1998; Tamura et al., 1998; Kim et al., 2000; Qiao et al., 2000).

Concept of bystander effect

An important component of this model of gene therapy is a bystander effect - a tumor regression after treatment with GCV which is greater than that due to the death of the TK-transduced tumor cells alone. It was noticed that neighboring, bystander tumor cells that do not express HSV-TK are also killed (Moolten and Wells, 1990; Culver et al., 1992; Freeman et al., 1993; Ram et al., 1997). Bystander cell death is not limited to the HSV-TK/GCV treatment paradigm because the bystander effect is also found in other gene/prodrug systems (Dilber and Gahrton, 2001). Many of the bystander cell killing studies have indicated that a tumor cell transduction rate of 10 to 20 percent, or even less, can result in significant reduction in non-transduced tumor cells. In vivo tumor eradication through the bystander effect has sometimes been reported in animal models (Culver et al., 1992; Freeman et al., 1993; Kuriyama et al., 1999), but in practice, tumor eradication has not been seen in the clinical trials utilizing the HSV-TK paradigm. This is so because much of the tumor mass in human gliomas escape HSV-TK infection, and the amount of bystander cell death is not as great as that seen in experimental tumor models (Ram et al., 1997;

Rainov, 2000). Lam and Breakefield (2001) have pointed out several relevant differences between tumor in humans and animals, noting that animal tumors are typically smaller and less invasive than those found in humans. Nevertheless a bystander effect acts to amplify the prodrug activation therapy. This observation has been much studied both *in vitro* and *in vivo* in the experimental literature, because a clear understanding of bystander cell killing might lead to greater clinical utility for tumor regression or, at least, an increase in the ability to eliminate those tumor cells which remain after surgical debulking (Katzmann et al., 1998; Marconi et al., 2000).

Mechanisms of the bystander effect

The limited conditions of an *in vitro* assay usually allow for a single mechanism to be implicated in bystander cell death, but there are several mechanisms hypothesized whereby non-TK-transduced cells are killed in vivo. One suggestion is that apoptotic factors released into the extracellular space by dead or dying cells are phagocytosed by the non-TK-transduced healthy bystander cells, inducing their death (Freeman et al., 1993; Bai et al., 1999). A second mechanism is proposed to occur due to in situ TK-transduction of brain capillary endothelial cells, which are killed after exposure to the prodrug, thereby reducing blood supply to the tumor (Ram et al., 1994, 1997). A third mechanism involves the stimulation of an immune response. Histological examination of tumors from experimental animals shows evidence of cytokine release following the HSV-TK/GCV treatment (Freeman et al., 1995; Ramesh et al., 1996) and infiltrates of lymphocytes and macrophage (Barba et al., 1994; Gagandeep et al., 1996; Bi et al., 1997; Di Meco et al., 1997; Kruse et al., 1997; Dewey et al., 1999; Kuriyama et al., 1999; Okada et al., 2001). Other evidence of the involvement of the host immune response comes from tumor immunized animals given HSV-TK/GCV treatment (Ramesh et al., 1999); they exhibited anti-tumor reactions distant from the site of treatment (Kianmanesh et al., 1997; Pope et al., 1997; Kruse et al., 2000; Dilber and Gahrton, 2001). Perhaps the most convincingly-demonstrated mechanism relies on earlier findings of the metabolic communication between cells (Subak-Sharp et al., 1969; Fujimoto et al., 1971; Dickerman and Tischfield, 1978) and implicates cell-to-cell contact and the formation of gap junctions between cells to effect the transit of the toxic phosphorylated GCV metabolites from the HSV-TK-transduced cells into neighboring, non- TK-transduced cells (Bi et al., 1993; Wu et al., 1994; Kruse et al., 2000; Burrows et al., 2002).

Of the alternate explanations for bystander cell death in vivo, none have been rejected, and it seems reasonable that the in vivo cell killing may be due to multiple mechanisms. For example, it is thought that in certain cell types one mechanism might be active in bystander cell death, while in other cell lines a different mechanism(s) might predominantly function (Denning and Pitts, 1997; Princen, et al., 1999). More integrative interpretations now support the concept that the bystander effect seen in vivo may be the result of more than just one mechanism, for the simple fact that, unlike in vitro experiments, each of the four mechanisms is available to contribute some portion of the total bystander cell killing (Gagandeep et al., 1996; Freeman 2000).

Characteristics of the bystander effect

It is clear that a dramatic bystander effect can be achieved in vitro. Typically small percentages of HSV-TK transduced cells can result in very large reductions of neighboring cell numbers after exposure to GCV (Kruse et al., 1997, 2000; Robe et al., 2000; Burrows et al., 2002). Several reports also indicate that substantial bystander cell death is exhibited in certain cell lines, but is weak to nonexistent in others (Fick at al., 1995; Wu et al, 1994; Ishii-Morita et al., 1997; Touraine et al., 1998a; Princin et al., 1999; Kawamura et al., 2001). Burrows et al. (2002) have shown that among human and murine glioma, melanoma and colorectal cancer cell lines, it is the gliomas that tend to manifest the strongest bystander cell death. Nevertheless other evidence suggests that there is variability in bystander cell death among different glioma cell lines. Within a sample of six human glioblastoma cell lines, 20% mixtures of TK-transduced with 80% wild type cells required a range in GCV concentrations up to as large as 730 mmol/l to achieve a lethal dose point of 50% (Sturtz et al., 1997).

Interestingly, bystander cell death is observed in xenogeneic cell mixtures, co-cultures of TK- and non-TK-transduced cell lines from different species, when exposed to GCV. It seems reasonable to conclude that species-specific differences, per se, are not particularly important in conferring GCV sensitivity to neighboring cells, although it is not entirely clear what factors contribute to differential bystander cell death in xenogeneic cell mixtures. In one report it was possible to identify both murine and human cell lines that showed high bystander cell death, and also human melanoma and leukemia cell lines that were resistant (Ishii-Morita et al., 1997). When cells were co-cultured in a xenogeneic mixture, it was not cell species that determined bystander cell death; rather, it was whether the TK-transduced cell line was bystander resistant or bystander sensitive. The same effect was demonstrated in vivo after subcutaneous injection of xenogenic cell mixtures into nude mice and examination of tumor incidence. Furthermore, Burrows et al. (2002) showed that cell mixtures of murine glioma lines with human melanoma cell lines produced a bystander effect that was characteristic of the level of bystander cell death shown by the non- TK-transduced cell line. Other examples of cross species bystander cell death have been reported by Wu et al. (1994), Freeman et al. (1995), Vironis et al.

(1997), and Carrio et al. (1999).

The gap junction hypothesis

Whether a gap junction independent mechanism for the *in vitro* transfer of toxic GCV metabolites leading to bystander cell death exists remains a question. Such a mechanism is thought to be some type of transfer of toxic metabolites through the extracellular space, and there is evidence that bystander cell death might occur under some in vitro circumstances which do not appear to be gap junction mediated (Denning and Pitts, 1997; Boucher et al., 1998; Du et al., 1998; Drake et al., 2000). Nevertheless it is gap junction intercellular communication (GJIC) which has emerged as the primary mechanism for the bystander cell death seen in the HSV-TK gene/prodrug system. Evidence supporting the gap junction hypothesis is found in the necessity of close contact between the HSV-TK cells and the neighboring cells, as demonstrated in cases where cell mixtures are incubated in high or low density cultures (Bi et al., 1993; Kruse et al., 2000; Burrows et al., 2002), or raised under conditions specifically designed to minimize cell contact (Mesnil et al., 1996; Yang et al., 1998; Robe et al., 2000). Cell-to-cell contact implies the formation of gap junctions, and in fact, it is thought that bystander cell death does not occur in the HSV-TK/GCV paradigm for cells that do not form gap junctions (Denning and Pitts, 1997). This latter point was demonstrated when Touraine et al. (1998a) sorted 17 tumor cell lines by the level of bystander cell death observed, and then tested each cell line for GJIC capability by calcein dye transfer. Five of the tumor cell lines showed no dye transfer, and in the same five cell lines there was no bystander effect.

More evidence implicating GJIC in bystander cell death comes from pharmacological manipulations of intercellular gap junction function. For example, in a variety of murine and human cancer cells it is possible to expose cells to a chemical agent that induces an increase in GJIC, and to observe that increase by means of dye transfer between cells. When murine adenocarcinoma cells were exposed to apigenin or lovastatin in the HSV-TK/GCV treatment paradigm, they showed an increase in bystander cell death, both in vitro and in vivo (Touraine et al., 1998b). Retinoic acid also increased GJIC and *in vitro* bystander cell death in a murine mesothelioma cell line. In the same report *in vivo* flank tumors were reduced by the HSV-TK/GCV treatment if retinoic acid was injected along with GCV (Park et al., 1997). It is also possible to chemically block or facilitate GJIC capability in rat C6 glioblastoma cells. When GJIC was chemically blocked in HSV-TK-transduced and non- TK-transduced C6 cells, bystander cell death was inhibited. Conversely, when GJIC was chemically facilitated, bystander cell death was increased (Robe et al., 2000). Thus, it seems clear that intercellular gap junctions can and do mediate bystander cell death in the HSV-TK/GCV paradigm.

Connexins

Phosphorylated GCV cannot pass through the plasma membrane except when traversing to neighboring cells by gap junctions. Intercellular gap junctions are cell membrane channels that connect adjacent cells. They are formed by a family of proteins called connexins and are important factors in bystander cell death. While gap junctions are only one form of cell-to-cell communication, they have an essential role in the transfer of molecules from the cytoplasm of one cell into an adjoining, coupled cell (Sosinsky, 2000). Each connexin protein originates from a distinct gene, and it is described by its molecular mass, which ranges in presently known connexins from roughly 26 to 60 kDa. Connexins are the protein subunits of the gap junctions between adjoining cells in many, but not all, mammalian tissues. It is known that many tissues express more than one form of connexin. The various possibilities of connexin combinations implies that metabolic coupling of cells is complex, in that it need not depend on only one type of connexin (Brink et al., 2000; Elenes and Moreno, 2000; Ruch, 2000).

Connexin mutation has been definitively linked to various hereditary diseases (Abrams and Bennett, 2000); however, some believe that connexin mutation is not a major factor in malignancies because it is rare in human cancers (Duflot-Dancer et al., 1997; Yamasaki et al., 1999). The specific changes in connexin expression that underlie cancer are not known because benign tumors and some malignant tumors appear to show stable connexin expression. Comparisons of malignant and non-malignant tissues have not led to a simple model of connexin expression in neoplasms, although in breast cancer and adrenocortical tumors, connexin gap junctions are believed to be characteristically decreased (Wilgenbus et al., 1992; Laird et al., 1999; Murray et al., 2000).

Connexins in the central nervous system

Various connexin (Cx) proteins are associated with cell-to-cell connection in the central nervous system (CNS). Astrocytes are served primarily by Cx43 gap junctions, but five other connexins may also be found. Neuron connectivity is associated with Cx36 and Cx43, and oligodendrocytes are connected by Cx32 and Cx45 (Spray et al., 2000). Brain astrocytes and glioma cells are characterized as highly coupled through gap junctions, while neurons and oligodendrocytes are sparsely coupled. Gap junctions are readily formed between primary cultures of human malignant glioma or glioma cells lines and co-cultured normal astrocytes. Connexin proteins play an important role in gap junction communication between astrocytes and glioma because the extent of bi-directional calcium signaling is a direct function of the Cx43 level in the glioma cells. Cultures of coupled rat astrocytes and Cx-expressing glioma cells result in smaller astrocytic cells, suggesting that gap

junction coupling with the glioma cells changes the astrocytic phenotype (Zhang et al., 1999).

The most common connexin found in primary tumors of the CNS is Cx43. The expression of Cx43 can be quite variable, especially in high-grade astrocytomas, compared to normal brain tissue. In glioblastoma cell lines three forms of Cx43 were found that differed slightly in molecular mass and probably represented differing amounts of phosphorylation of the protein; this in turn may be related to the localization of the connexin in the cell. Phosphorylated Cx43 tends to be found near the membrane where it is correlated with gap junction activity, and the unphosphorylated forms tend to have a more intercellular localization (Shinoura et al., 1996). Other primary brain tumors such as oligodendrogliomas, gangliogliomas, meningiomas, and medulloblastomas also express Cx43. Another connexin, Cx26, may be found in primary brain tumors where its expression is also quite variable (Estin et al., 1999). Thus, it is clear that connexin proteins are present in both normal and malignant brain tissues, but the variability in connexin levels and types seen in primary brain tumor cells suggests that many tumors may not have a homogeneous outcome to HSV-TK prodrug activation therapy. Improvement of the gap junction connectivity in brain tumors through experimental increase in the level of connexin expression might result in an enhanced bystander cell death for tumors (Shinoura et al., 1996; Estin et al., 1999; Burrows et al., 2002).

Implications of connexin expression

The primary role of connexins in malignant progression appears to be suppression of tumor formation and growth. Connexins are implicated in the regulation of cellular growth control through gap junctions (Rose et al., 1993). This fact supports the idea that defective gap junction intercellular communication contributes to neoplasia (Lowenstein and Kano, 1966). Nevertheless, the various connexin proteins are not necessarily equivalent in this regard, since in several cell types growth control appears to be a function of a particular endogenous connexin (Yano et al., 2001).

For malignancy in the CNS, research supports a role for Cx43 as a tumor suppressor. A decrease in the level of Cx43 expression was linked to the progression of malignancy in human gliomas (Huang et al., 1999). Further, the levels of Cx43 expression in human glioblastoma and neuroblastoma cell lines are decreased, but transfection of Cx43 into human glioblastoma cells reduces tumor cell proliferation in vitro as well as in nude mice (Cirenei et al., 1998). As measured by Lucifer yellow dye, however, tumor suppression resulting from Cx43 transfection did not appear to result from an increase in GJIC (Huang et al., 1998). Generally similar results were reported for connexin transfection into rat C6 and 9L glioma tumor cells (Naus et al., 1992; Princen et al., 2001). Other research has shown that transfection of human glioblastoma cells with Cx43 imparted an increased sensitivity to chemotherapeutic agents and a susceptibility to apoptosis. Yet the Cx43 mediated apoptosis following exposure to chemotherapy agents may not be related to any effect of gap junctions, since Cx43 transfected glioblastoma cells expressed only unphosphorylated Cx43 (Huang et al., 2001) and it is the phosphorylated forms of Cx43 that are likely involved in GJIC (Hossain et al., 1998).

Interestingly, Cx43 transfection into human glioblastoma cells also acts to decrease levels of Bcl-2. It was hypothesized that such reductions might underlie cell apoptosis in response to chemotherapeutic agents in cells transfected with Cx43 (Huang et al., 2001). If connexin expression does serve to regulate Bcl-2 levels then this fact could have important consequences. For example, bystander cell death was reduced by Bcl-2 transfection by Hamel et al. (1996), who suggested that tumors with high expression of Bcl-2 might show resistance to HSV-TK/GCV therapy. Thus, the possibility that connexins act to regulate other factors suggests that while connexin expression is involved with a growth control function for brain tumors, nevertheless, there is also some consideration that this role may be independent of GJIC and different from those actions of connexins which are specifically related to gap junctions (Duflot-Dancer et al., 1997; Mesnil et al., 1997; Yamasaki et al., 1999; Mesnil and Yamasaki, 2000).

Connexins and the bystander effect

Connexin proteins have been shown to mediate increases in bystander cell death, such as the increased in vitro bystander effect seen after the introduction of both HSV-TK and Cx43 into glioblastoma cells (Shinoura et al., 1996; Estin et al., 1999). Certain carcinoma cells that express little or no connexin have proven to be valuable experimental models for evaluating the importance of connexins in bystander cell death through cell-to-cell communication. For example, HeLa cells within the HSV-TK/GCV *in vitro* paradigm do not support bystander cell killing; however, if the HeLa cells are transfected with Cx43, then substantial bystander cell killing is found. Presumably the bystander effect in this model is mediated by connexin-induced cell-to-cell communication, which permits transfer of toxic GCV phosphorylated molecules from the HSV-TK cells to the neighboring, non- TK-transfected cells. This is so because cells co-cultured in the same medium, but lacking cell-to-cell contact did not display bystander cell killing (Mesnil et al., 1996). Duflot-Dancer et al. (1998) demonstrated in vivo that bystander cell death in HeLa cells was due to Cx43 transfection. In later work Tanaka et al. (2001b) refined the Cx43 transfection model to show that while no GJIC is found between *in vitro* mixtures of wild type HeLa cells, GJIC does form between mixtures of Cx43 transfected and wild type HeLa cells. That is, Cx43 is necessary in only one set of cells, not both. The GJIC will support a bystander effect, and various in vitro co-cultures of wild type HeLa cells

with Cx43 and/or TK-transfected HeLa cells indicated that better bystander cell death was obtained if the TK-gene and the Cx-gene were not co-expressed in the same cells. Tanaka et al. (2001b) hypothesized that the differential bystander effect they obtained was because of differential GJIC between cell groups. Nevertheless, their data may be relevant to the present review in that the maximum bystander cell death was found when Cx43 was transfected in HeLa cells (i.e. non-TK-transfected cells) that were later mixed with both TK-transfected cells and wild type cells.

A second connexin has also been implicated in bystander cell death in HeLa cells. Interestingly Cx26, and not Cx43, is believed to inhibit tumorigenicity of transfected HeLa cells, but it also supports a strong bystander cell death when transfected into HeLa HSV-TK cells. These data showed that a GJIC inhibitor blocked the bystander effect mediated by Cx26 transfection, thereby giving additional support to the idea that cell-to-cell communication through gap junctions underlies bystander cell death (Mesnil et al., 1997). The exogenous introduction of Cx26 was shown to increase bystander cell death in Cx26 low expressing human bladder cancer cells (Tanaka et al., 2001a) and in certain pancreatic cell lines (Carrio et al., 2001).

Data from another connexin low expressing cell line also supports the contention that the level of bystander cell death is related to the level of GJIC. Elshami et al. (1996) transfected a non-Cx43 and non-Cx32, but Cx45 low expressing human hepatoma cell line with either Cx32 or Cx43 and showed that there was a resulting increase in GJIC as measured with a double dye transfer technique. After transduction with an adenoviral vector containing HSV-TK and later exposure to GCV, the Cx43 transfected hepatoma cells demonstrated an increase in *in vitro* bystander cell death when compared to non-transfected hepatoma cells. Next, the Cx32 transfected hepatoma cells were compared to Cx32 transfected hepatoma cells exposed to tetracycline, which inhibits Cx32 transcription. The results showed that the bystander cell death was significantly less in the tetracyline-inhibited cells.

With respect to cells found in the CNS, rat C6 glioblastoma is a low GJIC cell line, although it can support a substantial bystander cell death (Dilber et al., 1997; Burrows et al., 2002). When C6 glioblastoma cells were transfected with Cx43, there was a resulting increase in both *in vitro* and *in vivo* bystander cell death. The authors asserted that the transfected Cx43 expression was correlated with intercellular coupling. In that study it was possible to group C6 clones which displayed moderate or high levels of Cx43; the bystander cell death in the C6 clones correlated with the level of transfected Cx43 (Dilber et al., 1997).

There is evidence that Cx43 expression does not always enhance bystander cell death in low bystander effect cells, even if they naturally express the connexin. In human colon tumor cells lines, for example, some lines express Cx43 diffusely in the cytoplasm, rather than on the cell surface where gap junctions can be formed. In such cell lines transfection with Cx43 may not result in functional gap junctions; we can only speculate at this time that this may be related to the phosphorylation state of the connexins. For instance, the HCT-8 and HT-29 human colon tumor cell lines did not manifest bystander cell death before or after Cx43 transfection. However, another human colon tumor cell line that did show enhanced bystander cell death after transfection with Cx43 was shown to express Cx43 at the cell surface (McMasters et al., 1998).

In an *in vitro* bystander cell death assay, co-cultures of non- TK-transduced and HSV-TK transduced cells are usually assumed to make gap junction connections by virtue of connexin proteins in each set of cells; however, certain authors have favored one set of cells for determining the degree of bystander cell death obtained. The data of Marconi et al. (2000) showed that it was the TK-positive cells that were key to determining the level of bystander cell death in the HSV-TK/GCV treatment system. Cells expressing little endogenous connexin benefited greatly by connexin co-transduction. Marconi et al. (2000) contrasted the effect of Cx43 transduction into high and low bystander effect cell lines *in vitro*. After Cx43 co-transduction into HSV-TK-transduced cells, both U-87MG glioblastoma and L929 fibrosarcoma cell lines showed increased bystander cell death, but the initially low bystander effect fibrosarcoma cells demonstrated the greater increase. The authors suggested that connexin expression by the TK-positive cells was responsible for the increased bystander cell death. To examine the level of bystander cell death obtained by varying the amount of connexin protein in the HSV-TK-transduced and non- TK-transduced cell lines, Marconi et al. (2000) conducted mixing experiments by co-culturing the U-87MG glioblastoma and L929 fibrosarcoma cell lines, both with and without Cx43 co-transduction in the HSV-TK cells. Without Cx43 co-transduction the bystander cell death was greater for both the glioblastoma and fibrosarcoma cell lines if the co-cultured TK-transduced cell line was the U- 87MG glioblastoma high bystander effect line. With Cx43 co-transduction, bystander cell death was increased in both non- TK-transduced cell lines to approximately the same level. The beneficial effect of Cx43 co-transduction with the HSV-TK gene followed by GCV was also demonstrated in vivo by increased survival times of animals with both U-87MG glioblastoma flank tumors and with intracranial tumors.

Contrary to the above, Namba et al. (2001) asserted that it was the connexin level in the non- TK-transduced cells that determined the amount of bystander cell death. Western blot analysis was used to show that endogenous Cx43 expression was high in 9L gliosarcoma cells, but low in C6 glioma cells. Transduction of Cx43 into C6 cells increased the connexin expression level, yet it was still about three times lower than the endogenous Cx43 expression in the 9L cells. Cell line mixing experiments *in vitro* and *in vivo* using the C6 and 9L rat cell lines

showed that bystander cell death was decreased when wild type C6 cells were co-cultured with either C6 or 9L TK-transduced cells. With either C6 or 9L TK-transduced cells, bystander cell death was greater when the Cx43 high-expressing 9L cells were used as the non-transduced cell line. Bystander cell death also was increased when Cx43 transduced C6 cells were co-cultured with TK-transduced C6 cells with unaltered connexin expression.

Using the connexin low-expressing human colon tumor cell line HT-29, McMasters et al. (1998) were not able to demonstrate *in vitro* bystander cell death before or after Cx43 transfection. Failure to find bystander cell death in HT-29 cells has also been reported by Todryk et al. (2001). Nevertheless bystander cell death in HT-29 cells was described by Boucher et al. (1998). Additionally, in Burrows et al. (2002) we reported that transduction with Cx43 increased bystander cell death in GCV-treated cell mixtures, presumably by increasing GJIC, if connexin was present in the non-TK-transduced cells (i.e. HT-29-Cx43 / HT-29-TK mixtures produced better bystander cell death than HT-29 / HT-29-Cx43-TK mixtures); however, HT-29 / HT-29-Cx43-TK mixtures exhibited the same bystander cell death as mixtures of HT-29 / HT-29-TK. A still greater increase in bystander cell killing was seen with Cx-43 transduction into both non-TK-transduced and TK-transduced cells. That data supports the hypothesis that it is the non- TK-transduced cells that are important in determining the extent of bystander cell death.

Table 1. The percent of communicating cells determined by double dye transfer in various colorectal cancer cell lines.

CELL LINES	MEAN PERCENT OF COMMUNICATING CELLS
CaCo2	5.05
WiDr	8.60
Sw480	68.00
Sw827	0.60
Sw948	9.20
HT-29	1.60
LS174T	5.50
HCT-116	9.90
LoVo	1.40

New experimental findings

In a previous report (Burrows et al., 2002) we presented the percent of dye transfer in eight glioma cell lines (Mean=65.3, SE=8.0) and in nine colorectal cell lines (Mean=12.2, SE=7.1). In Table 1 the mean percent dye transfer for two observations in each of the nine colorectal cancer cell lines is listed. The group median dye transfer is 5.5%, and clearly, dye transfer in eight of the nine colorectal cell lines was far below that of the glioma cell lines, which had a median value of 65.1%. Assuming the percent of dye transfer is related to GJIC, one can conclude that all but one of the colorectal cancer cell lines shown in Table 1 can be characterized as having low cell-to-cell communication.

In Fig. 2 a schematic of the calcein dye transfer procedure to assess GJIC is shown, along with the percent of dye transfer, detected by flow cytometry, when wild type HT-29 colorectal cancer cells (WT) are paired with wild type HT-29 cells or Cx43 transfected HT-29 cells (Cx43). In comparison with the dye transfer between wild type HT-29 cells, transfer from green-labeled Cx43 transduced cells into red-labeled Cx43 transduced cells was more than five times higher;

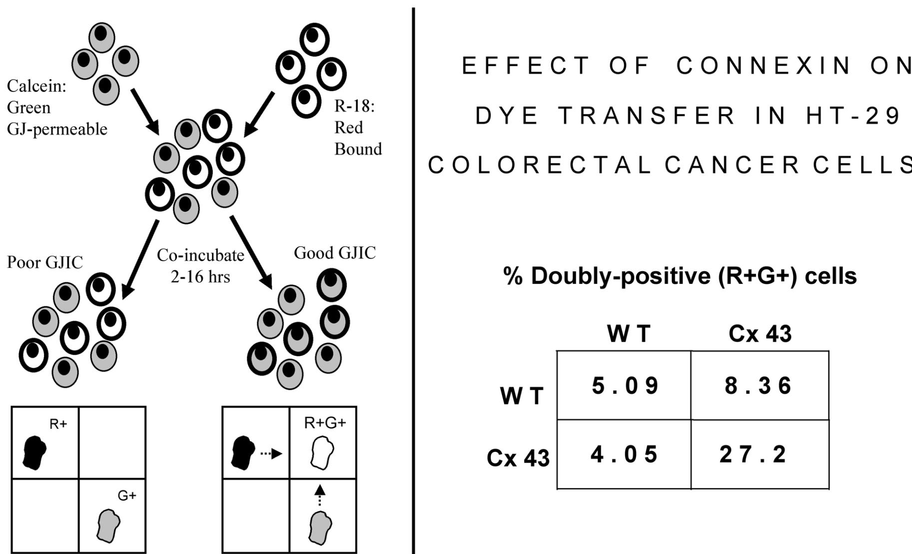

Fig. 2. Diagrammatic representation of the double-dye transfer technique to ascertain gap junction intercellular communication (GJIC) **(left panel)**, and the dye transfer obtained with co-incubates of wild type (WT) and connexin-43 (Cx43) transduced HT-29 cells **(right panel)**. Half of the cells to be monitored for GJIC are labeled with a gap junction permeable, cytoplasmic calcein green dye (shaded); the other half are labeled with a gap junction non-permeable, membrane-bound red dye (clear membrane-ringed). The cells are combined, and at time zero, when one can make the assumption that no dye transfer has occurred, the gates are set by flow cytometry. Then, after a period of co-incubation, flow cytometry is again performed on washed cells. The shift from single red (R+) or green (G+) positive cells to doubly-positive (R+G+) cells indicates the extent of gap junction communication established between the two groups of cells (left panel). The percent of dye transfer between co-incubates of HT-29 colorectal cancer cells, with or without Cx43 transduction, is presented after a 6 hr co-incubation (right panel). Cell-to-cell transfer of calcein dye became greater only when Cx43 was present in both the red- and green-labeled cells.

however, dye transfer was not facilitated if the transfer was from wild type cells into Cx43 transduced cells.

To demonstrate the effect of Cx43 transduction on bystander cell death in the suicide gene therapy paradigm, 5% HSV-TK-expressing (TK), or 5% HSV-TK- and Cx43-expressing (Cx43TK), HT-29 colorectal cancer cells were co-cultured *in vitro* with either wild type (WT) or Cx43-transduced (Cx43) HT-29 cells. The relative metabolic activity obtained after exposure of the cell mixtures to GCV is described in a bar graph (Fig. 3) where the effect of Cx43 transduction on bystander cell death can be readily compared to that obtained from the wild type and TK-transduced HT-29 colorectal cancer cells. The maximum bystander cell death resulted from Cx43 transduction into both of the co-cultured TK-transduced and non- TK-transduced cells. Transduction of Cx43 into either the non- TK-transduced cells or the TK-transduced cells also increased bystander cell death, however, the increase was greater when connexin was added to the non- TK-transduced cells.

In Fig. 4 we demonstrate that these observations hold at all of the percentages tested of TK-expressing co-cultures after GCV exposure. The amount of bystander cell death obtained in an *in vitro* metabolic assay of HT-29 colorectal cancer cells (WT) is plotted at various percentages of co-cultured TK-expressing HT-29 cells (TK). The figure displays the effect on bystander cell death when Cx43 is present in either the non- (Cx43) or the HSV-TK-transduced (Cx43TK) cells, or both. The maximum bystander cell death was always obtained with transduction of Cx43 into both TK- and non-TK-transduced cell groups. Similar to the effect depicted in Fig. 2, transduction of Cx43 into the non-TK-transduced cells also generally yielded a greater increase in bystander cell death than for the TK-transduced cells at the 5, 20 and 60% mixtures tested.

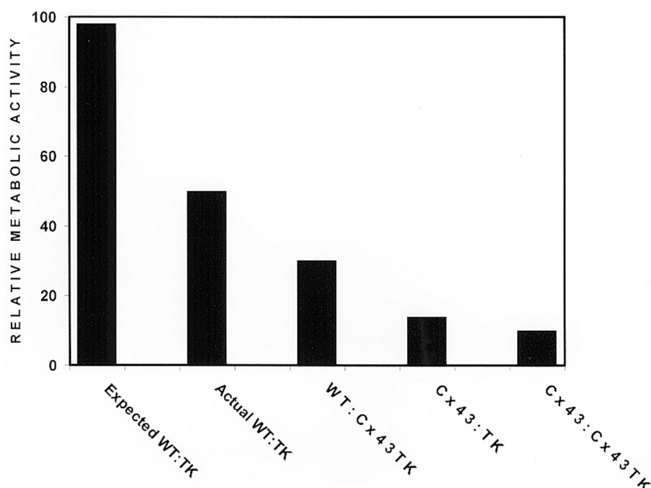

Fig. 3. *In vitro* bystander effect, determined from the relative metabolic activity obtained from ganciclovir treated cells after the co-culture of wild type HT-29 colorectal cancer cells (WT) with either 5% HSV-TK-expressing (TK) or 5% HSV-TK- and Cx43-expressing (Cx43TK) HT-29 cells. The reduction in metabolic activity, and therefore the increase in bystander cell death due to Cx43 transduction into either the TK-transduced cells, the non- TK-transduced cells (Cx43), or both, is depicted by the height of the bar assigned to each group of cells. The greatest reduction in metabolic activity was obtained when both the non- and TK-transduced cells also displayed Cx43. When only one of the populations displayed Cx43, the greater reduction in metabolic activity was obtained when the non-TK-transduced cells expressed Cx43.

Suicide gene therapy, connexins, and apoptosis

Programmed cell death or apoptosis is characterized by specific morphological alterations, which are visible by light microscopy in hematoxylin and eosin stained cells to the trained observer. Nuclear compaction and cytoplasmic condensation are observed, followed by breakdown of the nucleus into discrete fragments. Finally, cells break down into membrane-bound apoptotic vesicles in which cytoplasmic organelles appear to be intact (Gerschenson and Rotello, 1992).

We hypothesized that apoptosis could be increased in HT-29 cells with the addition of Cx43 to TK-transduced cells. We proceeded to determine the percentage of apoptotic cells by an *in vitro* quantitative morphologic assay 5 days after a 24 hr GCV treatment (10 μg/ml) of various mixtures of wild type (HT-29) and TK- (HT-29-TK) and/or Cx43-transduced (HT-29-CxTK or HT-29-Cx) cells. Apoptotic cell counts were obtained from hematoxylin and eosin stained monolayers of HT-29 colorectal cancer cells that contained either 20% TK-transduced, or TK- and Cx43-transduced, HT-29 cells when they were co-incubated with wild type or Cx43-transduced cells (Table 2). Counts were obtained of injured cells, readily visible from their appearance, which was strikingly and primarily of cells in late stage

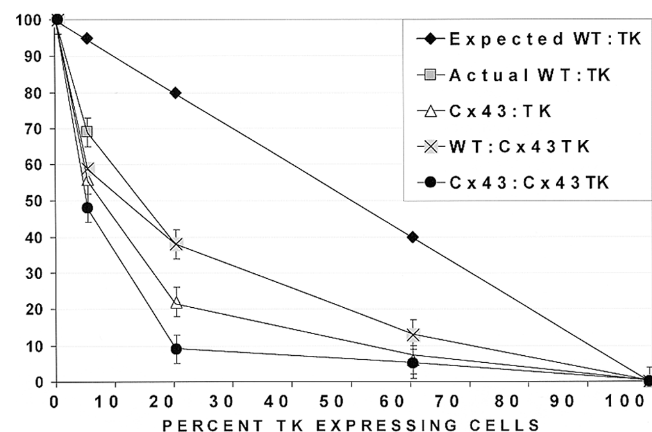

Fig. 4. The relative metabolic activity, as an indication of *in vitro* bystander cell death, was obtained with ganciclovir treated cultures of wild type HT-29 colorectal cancer cells (WT) or connexin-43 expressing HT-29 cells (Cx43) co-cultured with various percentages (5, 20, or 60%) of TK-expressing (TK) or TK- and Cx43-expressing (Cx43TK) HT-29 cells. The greatest reduction in metabolic activity at all of the percentages tested was when both the non- and TK-expressing cells also expressed connexin.

apoptosis with apoptotic bodies (Fig. 5, black arrows) or fragmented chromatin (white arrows); others had condensed nuclei (curved arrow). In this assay, the trend in apoptosis levels reflect that obtained for bystander effects when Cx43 is present in the non- or TK-transduced cell mixtures, or both. From the small differences in the apoptotic levels obtained (Table 2), however, one could not definitively make the conclusion that the data support a role for connexins in improving apoptosis in the suicide gene therapy treated cultures. In this type of assay only adherent cells are counted, leaving open the possibility that the results could be skewed since lysed cells from late apoptotic events would not be among those in the counts.

Discussion

The addition of connexin, a naturally occurring protein in many mammalian cells, to a low GJIC colorectal cancer cell line acts to increase cell-to-cell communication and the level of bystander cell death in the HSV-TK/GCV gene therapy paradigm. An increased calcein dye transfer, reflecting an increase in GJIC, in HT-29 cells due to Cx43 transduction is clearly supported by the data in Fig. 2. There is also a suggestion in the dye transfer data that cell-to-cell communication may be differentially sensitive to connexin protein added to the dye-transferring cell, rather than to the dye-receiving cell. This is evident because calcein dye transfer was not increased when Cx43 was added to only the dye-receiving cells.

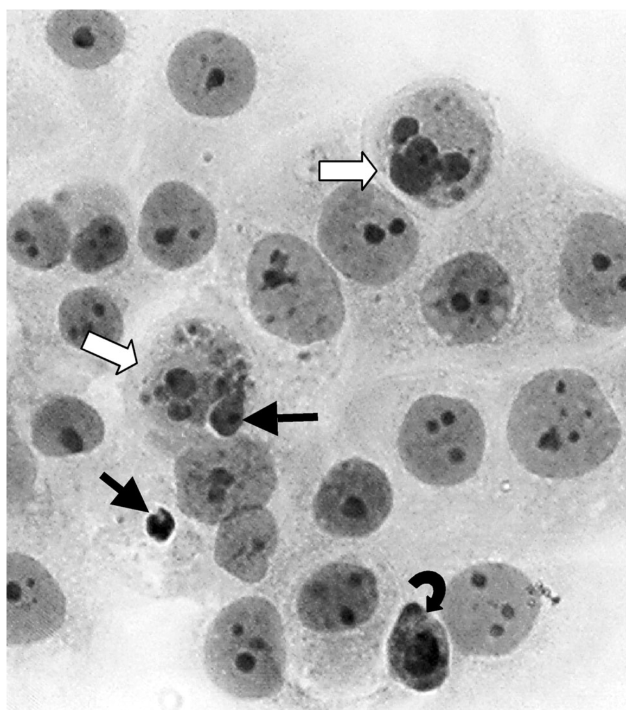

Fig. 5. In an *in vitro* quantitative morphologic assay, apoptotic HT-29 cells are visible in late stage apoptosis with apoptotic bodies (black arrows) and with fragmented chromatin (white arrows), while another cell is seen at an earlier apoptotic stage with a condensed nucleus (curved arrow). Normal looking cells are surrounding them. The apoptotic cells were present within a 20% TK-transduced mixed monolayer of wild type HT-29 and HT-29 TK-transduced cells after exposure of the cells to ganciclovir. The percentages of apoptotic cells obtained from cell counts of various HT-29 wildtype and TK ± Cx43 transduced cell mixtures can be found in Table 2.

With respect to the HSV-TK/GCV bystander effect, presumably the connexin-assisted increase in GJIC allows a more efficient transfer of toxic GCV metabolites between the TK-transduced cells and the non-TK-transduced cells, thereby increasing the amount of bystander cell death. If so, then combination therapy consisting of HSV-TK/GCV treatment along with connexin transduction may result in a more effective cancer treatment than with HSV-TK gene therapy alone.

While transduction of Cx43 into both TK-transduced and non-TK-transduced cells always resulted in the maximum bystander cell death, as described in Fig. 3, the data also suggest that the increase in toxic GCV metabolite transfer through connexin transduction is dependent on the addition of connexin to the non- TK-transduced cells, rather than the TK-transduced cells. This finding correlates with our new experimental data provided in this report, as well as our reported cell line mixing experiments (Burrows et al., 2002), which demonstrated that the level of bystander cell death obtained was determined by the non- TK-transduced cell line. Clinically, this is important since therapeutic approaches to increase intracellular levels of TK, or to increase the in situ transduction of TK, may not significantly enhance the bystander killing. However, combining suicide gene therapy with connexin gene therapy may synergize to improve bystander killing.

In summary, a review of connexin enhancement studies and the experimental data presented here support the notion that in tumor cells having a low GJIC, increasing the expression of gap junction proteins serves to increase the amount of bystander cell death and reduce tumor burden beyond what might be expected with suicide gene therapy alone.

Table 2. The percent of apoptotic cells by *in vitro* quantitative morphologic assay after ganciclovir treatment of 20% mixtures of TK-transduced (TK), or TK- and Cx43-transduced (Cx43TK), HT-29 cells mixed with wild type (WT) or Cx43-transduced (Cx43) HT-29 cells.

CELL COMBINATION WITH 30% TK POSITIVE HT-29 CELLS	% APOPTOSIS
WT / TK	15
Cx43 / TK	19
WT / Cx43TK	10
Cx43 / Cx43TK	21

Negative control of 100% wild type HT-29 cells exposed to GCV = 7% apoptotic count. Positive control of 100% HT-29 TK-transduced cells exposed to GCV = 37% apoptotic count.

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