Review

Bystander Effect in Herpes Simplex Virus-Thymidine Kinase/Ganciclovir Cancer Gene Therapy: Role of Gap-junctional Intercellular Communication

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Abstract

Antitumor suicide gene therapy is one of the emerging strategies against cancer. It consists of the introduction into cancer cells of a gene capable of converting a nontoxic prodrug into a cytotoxic drug. Because this therapeutic gene cannot be easily introduced into the whole cell population of a tumor, the successful eradication of tumors depends on a phenomenon called the “bystander effect,” by which the introduced gene can affect even cells in which it is not itself present. From a therapeutic point of view, it may be crucial to enhance this phenomenon through various means to achieve tumor eradication. One such suicide gene, the thymidine kinase gene from the herpes simplex virus, in combination with the prodrug ganciclovir, has been extensively and successfully used in some animal models exhibiting a strong bystander effect. Among the mechanisms involved in this phenomenon, gap junctional intercellular communication (GJIC) is directly involved in the transfer of the toxic metabolites of ganciclovir, which pass directly from herpes simplex virus thymidine kinase-expressing cells to surrounding cells that do not express it. Because GJIC appears to be a mediator of the bystander effect both in vitro and in vivo, here we review possible molecular strategies for enhancing the extent of tumor cell death by increasing the intratumoral GJIC capacity.

Introduction

Despite clear progress, conventional radiotherapeutic and chemotherapeutic treatments against cancer still place a burden on patients; their systemic administration can even lead to the appearance of secondary cancers (1, 2). Local strategies and better targeted weapons to destroy only tumor cells without affecting neighboring tissues can be developed using knowledge of carcinogenic processes. Some such emerging strategies make use of pharmaceutical drugs designed to block molecular pathways that are activated in cancer cells (3), the steps of carcinogenesis that make cancer cells invasive (4), or angiogenesis (5, 6). Strategies aimed at suppressing the expression of an oncogene or restoring the function of a defective tumor suppressor gene have already been studied experimentally, with the transfer of therapeutic genes into cancer cells to correct their genetic errors (7, 8). However, this kind of gene therapy can target one of several genes responsible for the malignant phenotype. Because no single gene has been shown to be altered in all human tumors, approaches to gene therapy that can operate independently of the genetic background of the cancer cells are therefore required. Such alternative gene therapies must focus on the eradication of the tumor cells. In particular, local and intratumoral chemotherapy can be achieved through gene therapy by inserting genes conferring drug sensitivity into the tumor cells, thus avoiding the systemic toxicity that occurs during conventional chemotherapeutic treatments. One such strategy involves “suicide gene therapy,” in which a gene coding for an enzyme that converts a nontoxic prodrug into a lethal compound is inserted into the tumor cells. Thus far, the most successful enzyme/prodrug combination tested in vitro and in animal models is HSV-tk, which has a high affinity for the prodrug GCV (9–12). This is an attractive approach because it targets the cell division process that is a characteristic of all tumor cells (13, 14).

To accomplish complete killing of a tumor, it was initially thought that the suicide gene HSV-tk would have to enter each individual cell. Given the low efficiency of current methods of gene delivery, this was not a realistic prospect. Fortunately, the HSV-tk/GCV strategy results in the death not only of the recipient (HSV-tk) tumor cells but also of surrounding nonrecipient (HSV-tk) tumor cells. This phenomenon is called the “bystander effect,” and it compensates for the low efficacy of vectors (viral or liposomal) in transferring genes into the tumor cells (15). Because gene transfer will probably remain a limiting factor for suicide gene therapy, it is crucial to induce a potent bystander effect. One approach is to enhance the local transfer of toxic metabolites from the transduced cells to the surrounding untransduced ones. A promising strategy is mediation of this transfer through GJIC. Propagation of toxic metabolites through GJIC was first observed 30 years ago (16), and we have shown its possible use in cancer therapy using an in vitro model (17).

Here we review recent progress made in the use of GJIC to enhance the bystander effect during cancer gene therapy mediated by the HSV-tk/GCV strategy.

The Molecular Mechanisms of the HSV-tk/GCV Therapy

HSV-tk (18) has a high affinity for acyclic analogues of deoxycytidine such as ACV and GCV; this affinity may explain the antitherapeutic and anticytomegalovirus activities of these drugs (19–21). The growth of cells infected with attenuated herpes simplex virus is inhibited by exposure to ACV or GCV, an action mediated by the HSV-tk gene of the viral genome (22–24). GCV is a better inhibitor of the growth of cells transfected with the HSV-tk gene than ACV, and, in particular, it is 10-fold more potent than ACV as an inhibitor of the growth of HSV-tk gene-transfected tumor cells (25).

The cytotoxic effect of guanosine analogues on cells expressing HSV-tk was demonstrated two decades ago (22). Its application to cancer therapy was first tested by transfecting BALB/c murine sarcoma cell lines with the HSV-tk gene. GCV treatment was sufficient to induce complete regression of palpable tumors that formed after injection of the transfected cells into mice (26). The effect of GCV
treatment was specific and did not induce resorption of HSV-tk−
tumors. After similar GCV treatment, this strategy also led to the
resorption of palpable tumors after s.c. injection of murine K2 sar-
coma or Ly18 lymphoma cells transduced with retroviral vectors
bearing the HSV-tk gene (9). Similar results were obtained with rat C6
glioma cells that were transduced with the HSV-tk gene before
injection into nude mice (27).

The cytotoxic effect of GCV results from its incorporation into
dNA by a process involving several steps, starting with its phospho-
rilation to form a monophosphate (ganciclovir monophosphate). Be-
cause its affinity for eukaryotic thymidine kinases is 1000 times
tower, GCV is essentially phosphorylated only by the viral enzyme
HSV-tk (23, 28). The ultimate product of further phosphorylation by
acellular kinases, GCV-triphosphate, competitively inhibits incorpora-
tion of the endogenous DNA precursor dGTP into DNA (29–31).
Moreover, the GCV-terminated strands of DNA are poor substrates
for DNA chain elongation (29), therefore the elongation of DNA
strands is prevented (30, 32, 33), leading to cell death. Apart from a
few possible exceptions with certain cell types (34), the death is
apoptotic and is probably independent of the p53 pathway (35–37).
Indeed, tumor cell lines exhibiting mutated p53 are also sensitive to
HSV-tk vectors, hence this therapeutic strategy can be applied irre-
spite of the p53 status of the tumors (38).

Other mechanisms of HSV-tk/GCV cytotoxicity also appear to
operate. Recent studies suggest that the high toxicity of GCV com-
pared with that of other substrates of HSV-tk such as ACV or
1-β-D-arabinofuranosylthymine is due not only to GCV triphosphate
but also to the incorporation of GCV monophosphate into the whole
genome (39). Moreover, independent of cell replication, some toxicity
of GCV to quiescent cells expressing HSV-tk, such as thyrocytes and
hepatocytes, has been reported (37, 40). The mechanism underlying
this phenomenon is not yet clearly understood but could be the
consequence of inhibition of mitochondrial DNA polymerase (37).
However, replicating DNA is considered to be the major target in
cancer cells for GCV activated by HSV-tk.

The HSV-tk/GCV Bystander Effect

The first in vivo experiments showed that systemic GCV treatment
can eradicate a tumor in which all cells express HSV-tk (9, 26, 27).
These experiments represented an ideal situation in the sense that all
of the malignant cells injected into the animals expressed the suicide
gene. However, in practical therapeutic approaches, only some of the
tumor cells can be reached in situ with a transgene.

One way to overcome the limited transfer of the suicide gene into
the tumor cells is to continuously produce in situ retroviral particles
carrying the HSV-tk gene. Such an approach, which is closer to
possible therapeutic applications, was tested by coinjecting fibrosar-
coma cells and 3T3 fibroblasts producing retroviral vectors carrying
the neomycin resistance (NeoR) gene (10). Analysis of the tumors in
vitro demonstrated that the NeoR gene was transferred to some (9%)
of the tumor cells, which thus became resistant to neomycin treatment
(10). The same strategy was used, replacing the NeoR gene with the
HSV-tk gene. Surprisingly, although the suicide gene transfer to the
umor cells was again much less than 100%, the tumors rapidly
regressed in most of the animals. The growth of s.c. injected tumor
cells of which 50% or even only 10% expressed HSV-tk could be
prevented by i.p. injections of GCV (10). This observation meant that,
contrary to the original expectation, in situ transfer of the HSV-tk
gene into a tumor could lead to its regression, even if the suicide gene
was not introduced into all of the tumor cells. It also suggested the
possibility of eliminating an established tumor by in situ injection of
a HSV-tk producer cell line. This was indeed observed in Fischer 344
rats carrying a 9L glioma previously implanted in a cerebral hemi-
sphere. Five days later, cells producing the HSV-tk retroviral vector
were injected intratumorally by stereotactic guidance. Subsequent i.p.
administrations of GCV induced complete tumor regression in nearly
all animals (10). When the β-galactosidase gene was used as a
transduction marker, in situ observation of the tumors showed that the
extent of transduction of the gene into the glioma cells varied greatly
(10–70%). This implies that cells that did not express the HSV-tk
gene became sensitive to GCV treatment even if 30–90% of the tumor
cells lacked the suicide gene (41). This phenomenon was not specific
to this cell line because the transfer of sensitivity to GCV from cells
infected with a retrovirus bearing the HSV-tk gene to neighboring
“naïve” cells has also been observed both in situ and in vivo with rat
C6 glioma cells (42).

GJIC as a Major Mechanism of the Bystander Effect

All of the in vivo studies described above have demonstrated
propagation of sensitivity to GCV from cells expressing HSV-tk to
nearby tumor cells that do not express it. Such a bystander tumor-
killing phenomenon (10) has been termed the “bystander effect” (43).

The involvement of GJs in the bystander effect was suggested (44,
45) because of the analogy between this phenomenon and the meta-
bolic cooperation assay that was described three decades ago (16).
This was an assay to estimate the extent of the GJIC capacity of cells
in culture by mixing cells deficient in HGPRT (HGPRT− cells),
which are unable to metabolize hypoxanthine, with normal cells (46).
The transfer of the toxic metabolite from HGPRT− cells to HGPRT−
cells was proven to be mediated by GJs (47), and the extent of toxicity
(“kiss of death”) in the coculture reflected the capacity of the cells
to communicate through GJs. The analogy between this kiss of death
phenomenon and the bystander effect was clear to Moolten (26), who
made the first observation of this effect in vitro, which was “presumed
to reflect transfer of GCV-P by metabolic cooperation between
HSV-tk positive and HSV-tk negative cells” (Ref. 26; see Fig. 1).

The hypothesis of metabolic cooperation to explain the bystander
effect was supported by direct observation of the transfer of toxic
derivatives of radiolabeled GCV from HSV-tk+ cells to HSV-tk−
cells (48). The implication of GJs in this transfer was based on the
known cell-to-cell transfer of phosphorylated nucleotides (16, 49).
GJs are intercellular channels that are made up from two juxtaposed
transmembrane hemichannels (connexons) provided by the adjacent
cells (reviewed in Ref. 50; see Fig. 2A). Each connexon is composed
of six Cx protein subunits with a central pore through which GCV-P
has appropriate size (M∞ <1000) (51) and properties to pass, as has
been shown for other nucleotides such as cAMP and ADP or other
second messengers (52, 53).

The mediation of the bystander effect by GJs is particularly inter-
resting because GJs seem to transfer toxic metabolites very efficiently.
For instance, it has been shown in three-dimensional cultures that only
1% of a tumor cell mass can significantly alter the growth of a mixture
of cells (HGPRT− and wild-type cells) in collagen gel containing
6-thioguanine, if they are extensively coupled (54).

In Vitro GJIC-mediated Bystander Effect

The first in vitro study to exploit GJIC to spread a cancer thera-
peutic effect was conducted in our laboratory over a decade ago (17).
This study demonstrated that diffusion of cytotoxic agents via GJIC
can improve chemotherapy efficiency. Because of the lack of GJIC
between normal and transformed cells, the killing was selective, and
only the transformed cells were eliminated (17). We later demon-
strated that GJs can also mediate a bystander effect in cancer gene
therapy in vitro by using HeLa cells, which exhibit very poor GJIC

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Fig. 1. In a tumor cell population, only a few cells can be reached by vectors carrying the HSV-tk gene. When those cells do express the tk gene (red nucleus), they become sensitive to GCV, which diffuses through the cell membrane. The HSV-tk+ cells are killed by the GCV-P they produce. A, because the GCV-P molecules cannot pass through the cell membrane, theoretically, only the HSV-tk+ cells (red cells) should be killed by GCV treatment. B, a direct diffusion from cytoplasm to cytoplasm of GCV-P could induce a bystander effect sufficient to eradicate a tumor cell population, even if only a few cells are HSV-tk+.

Fig. 2. Bystander effect in cancer gene therapy due to GJIC. Toxic GCV-P molecules can pass from a cell expressing the HSV-tk+ (tk+) gene to neighboring HSV-tk- tumor cells. A, the toxic GCV-P molecules (red arrows) pass from HSV-tk+ cells to HSV-tk- cells through the GJs. B, in HeLa cells, the bystander killing effect is observed only when cells expressing the HSV-tk gene communicate (as shown by the dye transfer assay on the left) through GJs. Cells expressing HSV-tk were mixed with their HSV-tk- counterparts at a 1:1 ratio. Similar results were obtained when only 10% of the cells were HSV-tk+.
capacity (Fig. 2B). When HeLa cells were transfected with an expression vector carrying the HSV-tk gene (HSV-tk\textsuperscript{+} cells), they became highly sensitive to GCV. If these cells were mixed with untransfected HeLa cells (1:1 ratio), GCV failed to eliminate the whole cell population. However, the outcome was quite different when we used HeLa cells that were able to communicate through GJs as a result of transfection of a gene coding for a common Cx, Cx43. Under these conditions, not only the HSV-tk\textsuperscript{+} cells but also the HSV-tk\textsuperscript{−} cells were killed by GCV, even if the culture contained only 10% HSV-tk\textsuperscript{+} cells (Fig. 2B). This bystander effect, by which the toxicity of GCV was transmitted to the remaining 90% of the culture, was clearly mediated by GJs because it was inhibited either by a lack of contact between the two cell types or by treating the cells with a long-term inhibitor of GJIC (55). These findings are supported by those from other laboratories reporting that the level of GJIC is predictive of (or correlated with) the extent of the bystander effect in vitro, whatever the origin of the cancer cell lines (Refs. 56–60; see Table 1). Indeed, it has been shown that cell lines exhibiting the bystander effect in vitro are able to transfer radiolabeled GCV between them if they do express Cxs (67). The type of Cx expressed (by transfection or otherwise) does not appear to be crucial for the bystander effect because similar results were obtained with HeLa cells expressing various Cxs such as Cx43 or Cx26 (55, 68) and in a mouse neuroblastoma cell line expressing Cx37 (Ref. 69; see Table 2).

Nevertheless, some in vitro work did not exhibit a close relationship between GJIC capacity and the extent of the bystander effect. This was observed on lung cancer cell lines in which the dye transfer assays exhibited a low GJIC capacity. The authors concluded from their results that basal GJIC would be sufficient to obtain an extensive bystander effect. However, the fact that they could not decrease the bystander effect by inhibiting the GJIC may suggest that the bystander effect was not completely mediated by GJs, even if cell-to-cell contacts were necessary (71). In some rare cases, the lack of intercellular contacts did not prevent the occurrence of the bystander effect. It is possible that in such cases, as with some rat colon adenocarcinoma cells, a diffusible factor is involved in the death propagation (61).

Similar conclusions were obtained with rat glioma cells in culture (72), even if GJIC was clearly involved in the in vivo situation (64).

Table 1  Correlation between GJIC and in vitro/in vivo bystander effects in various tumor cell types

<table>
<thead>
<tr>
<th>Species</th>
<th>Tumor</th>
<th>Cell line</th>
<th>Bystander effect</th>
<th>GJIC</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
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<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Colon adenocarcinoma</td>
<td>MCA38</td>
<td>+</td>
<td>+</td>
<td>60</td>
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<tr>
<td>Neuroblastoma</td>
<td>N2a</td>
<td>–</td>
<td>–</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>S49-1</td>
<td>–</td>
<td>–</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Gliosarcoma</td>
<td>9L</td>
<td>++</td>
<td>++</td>
<td>60</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>UMR106</td>
<td>++</td>
<td>+</td>
<td>60</td>
<td></td>
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<tr>
<td>Mesothelioma</td>
<td>Meso20</td>
<td>+</td>
<td>+</td>
<td>60</td>
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<tr>
<td>Colon adenocarcinoma</td>
<td>DHD/K12</td>
<td>+</td>
<td>+</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Pancreas</td>
<td>BXPC-3</td>
<td>++</td>
<td>+</td>
<td>59</td>
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<tr>
<td></td>
<td></td>
<td>PANC-3</td>
<td>+</td>
<td>59</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>MIA/PAC2</td>
<td>–</td>
<td>–</td>
<td>59</td>
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<tr>
<td>Mesothelioma</td>
<td>JMN</td>
<td>+</td>
<td>++</td>
<td>60</td>
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<tr>
<td>Colon adenocarcinoma</td>
<td>HT29</td>
<td>–</td>
<td>–</td>
<td>62</td>
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<td></td>
<td></td>
<td>HCT-8</td>
<td>–</td>
<td>–</td>
<td>62</td>
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<td>HCT-116</td>
<td>+</td>
<td>–</td>
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<td>Erythroleukemia</td>
<td>K562</td>
<td>–</td>
<td>–</td>
<td>60</td>
<td></td>
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<tr>
<td>Breast adenocarcinoma</td>
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<td>–</td>
<td>–</td>
<td>55</td>
<td></td>
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<td></td>
<td></td>
<td>MDA-MB435</td>
<td>–</td>
<td>–</td>
<td>61</td>
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<td><strong>In vivo (transplanted tumors)</strong></td>
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<tr>
<td>In rats</td>
<td>Colon tumor</td>
<td>DHDProB</td>
<td>+/-</td>
<td>+/-</td>
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<tr>
<td>In mice</td>
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<td><strong>In vivo (intrinsic tumors)</strong></td>
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<td>Human</td>
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<td>C6</td>
<td>–</td>
<td>+/-</td>
<td>64</td>
</tr>
<tr>
<td>In mice</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
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<td></td>
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<tr>
<td>Neu transgenic</td>
<td>Mammary tumor</td>
<td>+/-</td>
<td>+/-</td>
<td>66</td>
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**In Vivo GJIC-mediated Bystander Effect**

The bystander effect mediated by GJs that we reported in vitro has also been observed in vivo. The major conclusion from these in vivo studies is the same as that from the in vitro studies: that GJIC, as a mediator of the bystander effect, can compensate for a low number of HSV-tk\textsuperscript{+} cells in propagating the killing effect of GCV in an induced tumor. For instance, transfection of the Cx37 gene reduced the weight of tumors induced by mouse neuroblastoma cells by up to 60% when 50% of the cells expressed the HSV-tk gene (69). Expression of the Cx43 gene in rat C6 glioma cells induced a similar phenomenon in nude mice, even when only 5% of the injected cells were HSV-tk\textsuperscript{+} cells (64). However, the tumor-suppressive role of endogenous Cx43, which is expressed in parental nontransfected C6 glioma cells, may have interfered with the observed bystander effect (73). Moreover, in the above-mentioned studies, the mice were killed soon after the end of GCV treatment, preventing an estimation of the duration of the bystander effect mediated by Cxs in down-regulating or preventing the growth of the remaining part of the tumors.

We found that the growth of 10\textsuperscript{4} injected HeLa cells, which are highly tumorigenic, was prevented by 10% HSV-tk\textsuperscript{+} cells only if the cells expressed Cx43. These results indicate that Cx43 expression can efficiently induce the bystander effect such that the growth of tumors is inhibited even when 90% of the injected cells are resistant to GCV, reproducing in vivo what we observed in vitro. Even when GCV was administered when the tumors were already palpable, tumor growth was reduced by 66% or 77% (for mixtures containing 10% or 50% HSV-tk\textsuperscript{+} cells, respectively) compared with the growth of tumors induced by communicating but HSV-tk\textsuperscript{−} cells (65). The GJ-dependent bystander effect was so efficient that some of the mice were still alive more than 2 months after the GCV treatment. In contrast, with Cx43\textsuperscript{−} HeLa cells, GCV decreased the size of the tumors only in the same proportion as the percentage of HSV-tk\textsuperscript{−} cells present in the original mixtures.

**Increasing the GJIC Bystander Effect**

All of the data we have presented thus far show that GJIC mediates a strong bystander effect. Moreover, in most cases, there is a corre-
loration between a lack of GJIC capacity and the absence of a bystander effect (Table 1). When GJIC is present, the intercellular transfer of GCV occurs within a few hours (58). This rapid intercellular spread of the toxic metabolites within a tumor mass helps to increase the efficiency of a suicide gene transferred into a tumor and points to applications of GJIC in therapy of localized cancers (67). The extent of GJIC in tumors then becomes a decisive factor for the bystander efficacy. Unfortunately, most of the cancer cell lines we have tested exhibit such a low GJIC capacity that we considered this to be a phenotypic characteristic of cancer cells (Ref. 74; see Table 3). Such a lack of GJIC would decrease the bystander effect and thus prevent an efficient eradication of the tumors. In a few cases, we have indeed observed that cancer cells that did not exhibit a satisfactory bystander effect in vivo were GJIC-deficient in vitro. This was the case for GJIC-deficient mammary tumor cells obtained from neu transgenic mice; when the HSV-tk gene was transduced into 10% of the tumor cells, the bystander effect was insufficient to allow the eradication of the tumors by GCV (66). Similarly, with colon cancer cells injected i.p. into rats, the bystander effect was so low because of their poor GJIC capacity that 75% of the injected cells had to contain the transduced HSV-tk gene before any curative effect of GCV treatment was seen (63). The low expression of Cx43 was also related to the limited bystander effect observed in vivo for human medulloblastoma cells (81). It is therefore imperative to screen tumors for their GJIC and/or induce GJIC to obtain successful gene therapy with the help of the bystander effect.

Prediction of Bystander Effect by Screening GJIC-proficient Human Tumors

As a crucial parameter for HSV-tk gene therapy, the level of GJIC in situ in tumors should be predictive of the efficacy of the bystander effect (59). Although most cancer cell lines are defective for GJIC, we still know very little about GJIC capacity inside human tumors. Few in situ functional studies of GJIC in human tumors have been carried out, but the available data show that GJIC was absent or low in stomach carcinomas and hepatocellular carcinomas (Refs. 79 and 80; see Table 3). To assess whether certain tumor types would be more susceptible to HSV-tk/GCV therapy, we propose to screen human tumors for their GJIC ability. The direct ex vivo estimation of GJIC on tumor biopsies would be the best way of performing such screening (82). However, this approach would be limited by the availability of fresh tumor samples and the technical difficulty of performing such analyses in tissues that are difficult to microinject with dyes and/or are heterogeneous in their cell populations. A broader study could be performed by screening for the presence of Cxs in fixed tumor tissues, which are already available in existing banks of biopsies, using antibodies directed against some common Cxs (Cx26, Cx32, and Cx43), which have recently become commercially available. Such screening would also provide valuable information about the putative role of GJIC in human carcinogenesis (74). This indirect screening of tumors by immunolocalization of Cxs in precisely identified cancer cells has previously been carried out with some human samples (83, 84), and the results suggested that some human tumor types would be more likely to exhibit a GI-mediated bystander effect than others. The deficiency of originally expressed Cx43 GJs (85, 86) in breast ductal carcinomas and infiltrating lobular carcinomas (87) and also in high-grade prostatic adenocarcinomas (88) suggests that these tumors would not be a good target for the HSV-tk/GCV strategy. Similar observations have been made on human ovarian cystadenocarcinomas (89). The situation appears to be different for primary human brain tumors, in which both Cx26 and Cx43 are highly expressed (90). However, generalization of the results from one biopsy may be different for others taken from the same tissue because Cx expression may vary greatly from one sample to another, as observed for Cx43 in high-grade astrocytomas (91). However, according to our in vitro and in vivo data, the localization of GJs at cell-to-cell contact areas is more important than the Cx expression per se in predicting their function. In human hepatocellular carcinomas as well as in rat liver, we observed that defective GJIC capacity was related to the cytoplasmic localization of the Cxs in the cells (79, 92), and this has been observed to be associated with a poor bystander effect, independent of the level of expression of the Cxs (62).

Table 2 Increase of bystander effect by expressing exogenous (transfected) Cx genes in different cell lines

<table>
<thead>
<tr>
<th>Species</th>
<th>Tumor of origin</th>
<th>Cell line</th>
<th>Transfected Cx gene</th>
<th>GJIC</th>
<th>Bystander effect</th>
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<tbody>
<tr>
<td>In vitro</td>
<td>Mouse</td>
<td>Hepa 1-6</td>
<td>Cx43</td>
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<td>Neuroblastoma</td>
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<td>Cx37</td>
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<td>Adrenal</td>
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<td>Cx43</td>
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<td>Cx43</td>
<td>↑</td>
<td>↑</td>
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<td>HCT-8</td>
<td>Cx43</td>
<td>NT</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>HCT-116</td>
<td>Cx43</td>
<td>NT</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>HeLa</td>
<td>Cx43</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Cx43</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

Table 3 Examples of human carcinoma cell lines and tumors in situ with low GJIC capacity

<table>
<thead>
<tr>
<th>Cx</th>
<th>mRNA</th>
<th>Protein</th>
<th>Localization</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>None</td>
<td>+</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>Epidermis</td>
<td>+/+</td>
<td>+/+</td>
<td>Epidermis</td>
<td>76</td>
</tr>
<tr>
<td>Cervix</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>77</td>
</tr>
<tr>
<td>Liver</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>78</td>
</tr>
<tr>
<td>Bladder</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>79</td>
</tr>
</tbody>
</table>

Tumors in situ

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Loss</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>Cytoplasm</th>
<th>79</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>None</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup> Decreased GJIC capacity or Cx mRNA correlated with the malignant phenotype.
<sup>b</sup> No data.
<sup>c</sup> GJIC capacity was estimated by electrical coupling.
late the synthesis of the Cxs in tumors. However, these mechanisms
by Cx43 will be increased by treatment with cAMP or retinoids
However, the present evidence indicates that a basal GJIC mediated
whether it is a Cx- and/or cell type-specific phenomenon (Table 4).
GJIC needs to be further studied
in vitro
This has been observed both
of GJIC (or Cx expression) can lead to an enhanced bystander effect.5 This type of biochemical increase of
doubling of the bystander effect.5 This type of biochemical increase of
cancer. The HSV-tk/GCV strategy may still be applicable to GJIC-deficient
tumors, if one can increase the GJIC capacity
in situ
by treating tumor cell
in vivo
by specific chemical treatments (93). cAMP (94, 95), retinoic acids (96, 97),
carotenoids, which increase the dye coupling of human dermal fibro-
blasts but not of human keratinocytes (98). It may also be Cx type
specific; for example, glucocorticoids (dexamethasone and hydrocor-
Glucagon
Hepatocytes (rat)
Cxs2, Cx32 up-regulation
113
Retinoids
All-trans-retinoic acid
Kidney cells (dog)
Cxs43 up-regulation
114
Liver epithelial cells (rat)
Cxs43 up-regulation
115
Skin (rat)
Cxs26, Cxs43 up-regulation
116
Teratocarcinoma cells (mouse)
Cxs43 up-regulation
117
Retinol (vitamin A)
Filoblasts (mouse)
ND
118
Carotenoids
β-Carotene
Filoblasts (mouse)
Cxs43 up-regulation
120
Echininone
Cxs43 up-regulation
121
Cantaxanthin
Cxs43 up-regulation
121
Cryptoxanthin
Cxs43 up-regulation
121
4-Hydroxy-β-carotene
Cxs43 up-regulation
121
Lutein
ND
121
Lycopen
ND
121
α-Carotene
ND
121
Flavanoids
Flavanone
Liver epithelial cells (rat)
Cxs43 up-regulation
122
Apigenin
Cxs43 up-regulation
100
Tangeretin
Cxs43 up-regulation
100

Table 4  Examples of up-regulation of GJIC by chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Cell types</th>
<th>Cx regulation</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP (and derivatives 8-bromo-cAMP, dbcAMP)</td>
<td>Choriocarcinoma cells (human)</td>
<td>Cxs40 up-regulation</td>
<td>104</td>
</tr>
<tr>
<td>Thyroid cells (porcine)</td>
<td>NDa</td>
<td></td>
<td>105</td>
</tr>
<tr>
<td>Hepatoma cells (rat)</td>
<td>Cxs43 up-regulation</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Mammary tumor cells (mouse)</td>
<td>Cxs43, Cxs45 up-regulation</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Glioma cells (rat)</td>
<td>Cxs43 up-regulation</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Ventricular myocytes (rat)</td>
<td>Cxs43, Cxs45 up-regulation</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Lung epithelial cells (mouse)</td>
<td>Cxs43 up-regulation</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Normal and malignant prostate epithelial cells (human)</td>
<td>Cxs43 up-regulation</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Forskolin</td>
<td>Hepatocytes (rat)</td>
<td>Cxs32, Cxs26 up-regulation</td>
<td>99</td>
</tr>
<tr>
<td>Corticoids</td>
<td>Hepatocytes (rat)</td>
<td>Cxs32 up-regulation</td>
<td>99</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Hepatocytes (rat)</td>
<td>Cxs32, Cxs26 up-regulation</td>
<td>113</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>Kidney cells (dog)</td>
<td>Cxs43 up-regulation</td>
<td>114</td>
</tr>
<tr>
<td>All-trans-retinoic acid</td>
<td>Liver epithelial cells (rat)</td>
<td>Cxs43 up-regulation</td>
<td>115</td>
</tr>
<tr>
<td>Echininone</td>
<td>Skin (rat)</td>
<td>Cxs26, Cxs43 up-regulation</td>
<td>116</td>
</tr>
<tr>
<td>Cantaxanthin</td>
<td>Teratocarcinoma cells (mouse)</td>
<td>Cxs43 up-regulation</td>
<td>117</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>Filoblasts (mouse)</td>
<td>ND</td>
<td>118</td>
</tr>
<tr>
<td>4-Hydroxy-β-carotene</td>
<td>Cxs43 up-regulation</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>ND</td>
<td></td>
<td>121</td>
</tr>
<tr>
<td>Lycopen</td>
<td>ND</td>
<td></td>
<td>121</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>ND</td>
<td></td>
<td>121</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>Liver epithelial cells (rat)</td>
<td>Cxs43 up-regulation</td>
<td>122</td>
</tr>
<tr>
<td>Apigenin</td>
<td>Cxs43 up-regulation</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Tangeretin</td>
<td>Cxs43 up-regulation</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

a ND, no data at the molecular level.

Biochemical Induction of GJIC in Tumors

The HSV-tk/GCV strategy may still be applicable to GJIC-deficient tumors, if one can increase the GJIC capacity
in situ, e.g., by specific chemical treatments (93). cAMP (94, 95), retinoic acids (96, 97), carotenoids (98), glucocorticoids (96, 99), and flavanoids (100) have such an effect in vitro. This effect may be cell type specific, as with carotenoids, which increase the dye coupling of human dermal fibro-
blasts but not of human keratinocytes (98). It may also be Cx type
specific; for example, glucocorticoids (dexamethasone and hydrocor-
Glucagon
Hepatocytes (rat)
Cxs26, Cxs32 up-regulation
113

Some evidence already suggests that a chemically induced increase
of GJIC (or Cx expression) can lead to an enhanced bystander effect. This has been observed both in vitro and in vivo by treating tumor cell
clines with retinoids, apigenin, or lovastatin (101, 102). Similarly,
treatment of human breast adenocarcinoma cells with cAMP signifi-
cantly decreased the percentage of HSV-tk-transduced cells necessary for a 50% bystander effect (103). Likewise, doubling the GJIC of
Cx43-transfected HeLa cells by cAMP treatment also resulted in
doubling of the bystander effect.5 This type of biochemical increase of
GJIC needs to be further studied in vitro and in vivo to determine whether it is a Cx- and/or cell type-specific phenomenon (Table 4).
However, the present evidence indicates that a basal GJIC mediated by Cxs43 will be increased by treatment with cAMP or retinoids (Table 4).

The full induction of GJIC depends on the mechanisms that regul-
ate the synthesis of the Cxs in tumors. However, these mechanisms
are far from being well understood, mainly because the noncoding
regions upstream of the Cx genes, which regulate Cx gene expression, have not been fully analyzed. On the other hand, it appears that Cx expression can be modified because these genes have specific pro-
moter regions. For instance, cAMP consensus responsive elements have been found in the Cxs32 gene (123, 124). This is in agreement
with the Cxs2 gene expression induced in hepatocytes by cAMP treatment (125). The promoter region appears to be complex; sites for
hepatocyte nuclear factor 1, nuclear factor κB, and glucocorticoids have been identified in the 5′-untranslated region of the Cxs32 gene
(124). Such a complex promoter region is probably a common feature of most Cx genes because they are expressed in a time- and tissue-
specific manner in organisms (126–128). However, this complexity, in turn, enhances the possibilities for inducing the expression of a
specific Cx in a targeted cell population.

Even if we were able to target the expression of a specific Cx, the
overall effect, as with many other genes, would depend greatly on the
methylation status of the promoter region. Silencing of Cx gene
expression due to hypermethylation has been observed for the Cxs26,6
Cx43, and Cxs32 genes (129). Silencing of Cx gene expression by
hypermethylation could explain why only a few cases of induction of
Cx expression and function by biochemical treatments have been
reported.

Another critical aspect is whether the newly induced Cx functions
normally in a tumor. Cx proteins have to migrate through the cyto-
plasm to be inserted into the cell membrane as connexons, which must

5 M. Mesnil, unpublished data.

6 R. Singal, Z. J. Tu, J. M. vanWert, G. D. Ginder, and D. T. Kiang, personal com-
munication.

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Cxs Not Only Mediate the Bystander Effect But Are Also Tumor Suppressors

Most communication-defective cancer cells lose their tumorigenic capacity or exhibit down-regulated growth once they are transfected with the appropriate Cx genes. This has been observed with cell lines of many different origins, such as rat hepatoma, murine fibroblasts (chemically transformed C3H10T1/2 cells), HeLa cells, and rat glioma (C6 cells), human breast carcinoma, and rhabdomyosarcoma (138–140, 144–146). Interestingly, the tumor-suppressive effect is sometimes dependent on the Cx type (144) but is not necessarily dependent on the induction of GJIC (147, 148).

A priori, the induction of Cx expression in tumor cells could lead to two beneficial effects on tumor control: (a) bystander effect mediation; and (b) tumor suppression. However, a strong tumor-suppressive effect resulting from the induction of Cx expression might actually prevent efficient DNA incorporation of GCV-derived nucleotides by slowing down the cell cycle. The results from our studies in vitro suggest that these two phenomena are not entirely antagonistic because we have shown that Cx26, a tumor suppressor in HeLa cells, can mediate a satisfactory bystander effect in vitro (68). However, such antagonism may need to be considered in relation to GCV killing efficacy in the case of a strong in situ induction of Cx expression.

This is also an important issue for the pharmaceutical manipulation of GJs. Some chemical compounds known to induce GJIC may act as growth regulators. For instance, apigenin induces not only GJIC but also G1 cell cycle arrest (149). Such activity could limit the efficacy of GCV incorporation into DNA, although a recent in vivo study suggests that this is not the case (102).

Conclusions and Perspectives

GJIC increases the extent of the bystander effect observed with HSV-tk/GCV gene therapy both in vitro and in vivo. This anticaner strategy may therefore be best adapted to treatment of tumors in which the cells have a significant basal level of GJIC. GJIC cannot easily be measured in situ, but a predictive estimation can be made by screening tumors for the presence of GJs correctly localized at cell-to-cell contact areas.

Tumors that may be suitable targets for HSV-tk/GCV therapy can be chosen according to a few characteristics (Fig. 3). They must be accessible for the intratumoral injections of vectors carrying the HSV-tk gene. Their cellular structure should be homogeneous, with cells firmly in contact with each other. Depending on the Cx types expressed, it should be possible to increase the basal level of GJIC by appropriate treatments. We believe that this kind of therapy could be extended in the future to GJ-deficient tumors by artificially enhancing the in situ communication capacity of the tumor cells through appropriate chemical treatments, once the promoter regions have been clearly identified. Artificial induction of GJIC by direct transfer of Cx genes may be inefficient but should be further studied in view of the surprising bystander effect that was induced through this strategy in vitro.

The results of in vitro studies indicate that GJIC may have various benefits for HSV-tk-mediated gene therapy. Some studies have shown that the strength of the bystander effect is less dependent on the level of HSV-tk activity than the cell-to-cell communication capacity (59). Other studies have also shown that HSV-tk activity can increase Cx43 function in hepatoma cells (70). Enhanced communication might enable the HSV-tk-expressing cells to resist the GCV treatment for longer time periods by permitting efficient diffusion of the GCV-P metabolites to the surrounding cells. This phenomenon has been termed the “Good Samaritan effect” and, if confirmed, should increase the efficiency of such therapy (150).

---

7 T. Tanaka, H. Yamasaki, and M. Mesnil, unpublished data.
Cxs themselves not only mediate the bystander effect but also have a separate tumor-suppressive action (151). Thus, we have proposed that Cxs have a dual effect on tumor control (68). Whereas it is clear that Cx genes suppress the growth of many tumor cells, there is increasing evidence to suggest that increased Cx gene expression at later stages of tumor development may facilitate metastasis (152). Therefore, for tumor control, it may be prudent to use Cx genes together with therapeutic suicide genes.

Moreover, the Cxs may work synergistically with other mechanisms that may be involved in the bystander effect. For instance, the Cxs may help to amplify the disruption of angiogenesis that is observed in vivo. Indeed, transduction experiments carried out in situ with cells producing retroviral particles bearing a β-galactosidase gene in a 9L glioma have shown that some blood vessel endothelial cells were infected with the viruses and expressed the gene (41). Cells located within or adjacent to a tumor are the most mitotically active endothelial cells responding to angiogenesis factors released by the tumor, and they should be sensitive to GCV if they express the HSV-tk gene. In rats, a decrease in tumor vasculature has been observed after the initiation of GCV therapy in HSV-tk-transduced tumors (153). The disruption of angiogenesis was accompanied by diffuse necrotic changes associated with intratumoral hemorrhage (153). Therefore, the elimination of these transduced endothelial cells with GCV may result in ischemia of the tumor mass (41, 153). Because the endothelial cells are GJIC proficient and express particular types of Cxs [Cx37, Cx43, and Cx40 (154)], they are potential targets for a “vascular” bystander effect that should contribute to tumor eradication.

Another consequence of the induction of the bystander effect by GJs may be the increased inflammatory infiltrations that have been observed in a range of target organs of both rats and mice (155–159). This antitumor response, leading to central hemorrhagic tumor necrosis, follows the killing of neoplastic cells as a result of treatment with GCV (160).

This immune response is probably one of the mechanisms involved in the bystander effect because the bystander effect is usually inefficient in athymic nude mice, being only a transitory phenomenon if no more than 50% of the injected cells express HSV-tk and are thus sensitive to GCV (161). In contrast to athymic mice, total tumor regression can be obtained with normal mice even if only 10–20% of the cells express HSV-tk (162). In addition, the more tumor cells killed by HSV-tk/GCV, the better the immune response should become. Therefore, the induction of the bystander effect by GJs should in turn lead to an increase in the immune response.

The local involvement of the immune system in the bystander effect raises the possibility that distant- and long-term systemic antitumor responses may also be induced. It has been reported that the growth of untreated tumors (tumors with no cells expressing HSV-tk) in one flank of a mouse can decrease when tumors with cells expressing HSV-tk are treated on the other flank (163, 164). This distant bystander effect seems to be efficient enough to prevent the growth of colonies of both HSV-tk- and HSV-tk- rat colon cancer cells in the whole peritoneal cavity of rats. These results suggest that such local suicide gene therapy could be used as a kind of cancer immunotherapy because it may have an effect not only against the HSV-tk-expressing tumors but also against distant tumors that do not express the transgene and are disseminated in the same organ (155, 165, 166). This distant bystander effect suggests that a local HSV-tk/GCV gene therapy may also prevent the growth of disseminated tumors and possibly metastases (167).

In conclusion, we consider that a strongly induced local bystander effect mediated by GJs, by enhancing the death of the cells in the primary tumor and, in turn, enhancing the local inflammation process, should increase the systemic immune response that may also act efficiently against metastases.

Acknowledgments

We thank Dr. John Cheney for editing the manuscript and Chantal Déchaux for secretarial help.

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Bystander Effect in Herpes Simplex Virus-Thymidine Kinase/Ganciclovir Cancer Gene Therapy: Role of Gap-junctional Intercellular Communication

Marc Mesnil and Hiroshi Yamasaki


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