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Hopes and Disillusions in Therapeutic Targeting of Intercellular Communication in Cancer

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1. Introduction

Although they tend to develop some independence upon malignant transformation, tumor cells and tumors remain"social" moieties. In many steps during tumor progression, tumor cells' interaction with each other and with their microenvironment is an essential element in their survival, growth and progression. This dependence on cell-cell interactions provides an opportunity for therapeutic interventions. In addition to long range interactions through growth factors, cytokines and other released molecules, the cells use various structures to interact directly, including gap junctions (GJ), tight junctions, adherens junctions and desmosomes.

Gap junction intercellular communication (GJIC), is a process involved in the transfer of second messengers such as cAMP, cGMP, glutamate, NAD+, IP_3, glutathione, and Ca^{++} ions, between cells, through channel structures called gap junctions (GJ). It is involved in various biological functions including regulation of cell growth, cell differentiation, and maintenance of tissue homeostasis (Wei et al. 2004). Structurally, gap junctions are formed by two head-to-head opposing hexameric transmembrane channels called connexons or hemichannels contributed by two interacting cells (Yeager and Harris 2007). The building units of connexons are the connexin proteins (Cxs), which are tetraspan integral membrane proteins (Nakagawa et al. 2010).

Expression and functional analysis of connexins and GJIC revealed that, in general, they are lost in cancer (Kandouz and Batist 2010) and their restoration has tumor inhibitory effects, which led to the concept that this type of intercellular communication plays a tumor suppressor role. Consequently, it early became clear that restoring GJIC and connexin expression, using different chemical treatments or by gene transfer, can be used to inhibit tumor cell growth (Fernstrom et al. 2002).

GJIC and Cxs have also been suggested to be involved during metastasis, although this role is still largely unclear. For example, on one hand connexin43 (Cx43) affects angiogenesis *in vitro* and *in vivo,* via an effect on proteins such as the Monocyte chemotactic protein-1 (MCP-1) and Interleukin-6 (McLachlan et al. 2006) , although this effect seems GJIC-independent (McLachlan et al. 2006) . On the other hand, Cx43-mediated GJIC facilitates metastatic homing to the lung via increased adhesion to endothelial cells (Elzarrad et al. 2008). GJIC as

a result of overexpression of Cx43 in MDA-MET, an aggressive derivative of the metastatic breast cancer cell line MDA-MB-231, decreased cell invasion (Li et al. 2008a). Cx43 and Cx26 have been suggested to contribute to metastasis of breast cancer to the lymph nodes (Kanczuga-Koda et al. 2006).

However, although connexins and gap junctions are tightly associated, connexins are capable of functions of their own. The exact role of GJIC-dependent versus –independent functions of connexins is still ill-understood and sometimes even paradoxical (Mesnil et al. 2005) (Dbouk et al. 2009). For example, connexins seem to act as tumor suppressors as well as tumor facilitators in the breast (McLachlan et al. 2007). The above-mentioned role of Cx43 in angiogenesis seems GJIC-independent (McLachlan et al. 2006).

Visibly, more studies are needed to understand the complex role of GJIC and Cxs in cancer. This lack of information is a major obstacle to the full use of the therapeutic potential of Cxs and GJIC in cancer. Nevertheless, this obstacle didn't prevent from a attempting many creative and promising therapeutic strategies.

2. Connexins and GJIC in gene therapy: the bystander effect

A major limitation to cancer gene therapy is the often limited transfection efficiency of target cells. This is the specific aspect where the field of gap junctions has been particularly helpful, using a mechanism to amplify the cytotoxic signal originating from a limited population of target cells.

2.1 Bystander effect-mediated functions of connexins and GJIC

For the GJIC researchers, it quickly became clear that the ability of cells to transmit signaling moieties to their neighbors would offer an interesting opportunity. This strategy, based on a process called "bystander effect" (BE) (Figure 1), doesn't require the therapeutic agent to reach all tumor cells (van, I et al. 2002). Thanks to the BE, triggering the death process in a single cell could be amplified by transfer of the cytotoxic signaling molecules via the GJICs, resulting in similar changes and fate in interacting cells. A major mechanism of the BE involves direct gap junctional intercellular communication (GJIC) and changes in connexins' levels translate into changes in the BE potential (Asklund et al. 2003; Elshami et al. 1996; Yamasaki and Katoh 1988). Therefore, the BE is an important factor in the efficiency of cancer therapy (Mothersill and Seymour 2004), but its function requires direct intracellular contacts to undergo cytotoxicity. So far, a major application for the BE has been gene therapy. Many authors have shown a decade ago that the BE promotes the so-called "suicide gene therapy".

The first explorations of the BE therapeutic potential involved the use of enzyme/prodrug gene therapy approaches. In this therapy, target cells are made to express an enzyme that converts a prodrug inside the cell into the cytotoxic active drug that is transmitted to and kills the interacting cells. Two combinations of enzymes and prodrugs have been particularly tested: the bacterial cytosine deaminase (CD) with the antifungal drug 5-fluorocytosine (CD/5-FC) and, most widely, the herpes simplex virus thymidine kinase (HSVtk) with the antiherpetic ganciclovir (HSVtk/GCV) (Mesnil et al. 1996; Trinh et al. 1995). In the CD/5-FC system, CD converts 5-FC into the active cytotoxic form 5-fluorouracil (5-FU) (Mullen et al. 1992). While ganciclovir (GCV), a nucleoside analogue, is poorly metabolized by mammalian thymidine kinases, it is phosphorylated by the HSVtk and cellular kinases and thus converted into the nucleotide GCV triphosphate, a cytotoxic

drug (Chen et al. 1994). The later works by incorporating into and blocking replication of DNA in dividing cells, resulting in induction of cell death (Thompson 1999). The phosphorylated form of GCV will be transmitted to neighboring cells via GJIC. For example, transfection of tumor cells expressing Cx43 with HSVtk will allow GCV to kill target as well as by-standing cells (Mesnil et al. 1996). GJIC and connexins have been shown to be involved during the BE-based HSV-tk/GCV therapy (Dilber et al. 1997; Vrionis et al. 1997; Elshami et al. 1996; Fick et al. 1995; Mesnil et al. 1996). BE using the UPRT/5-FU system (uracil phosphoribosyltransferase (UPRT) of E. coli origin and 5-fluorouracil (5-FU)) was found to be correlated to the level of Cx43 and GJIC (Kawamura et al. 2001). The extent of the role of GJIC and Cxs in BE-mediated cytotoxicity is most certainly underestimated. Many experimental therapeutic strategies make use of the BE but the role of GJICs or Cxs in their mechanism of action has not been investigated yet.

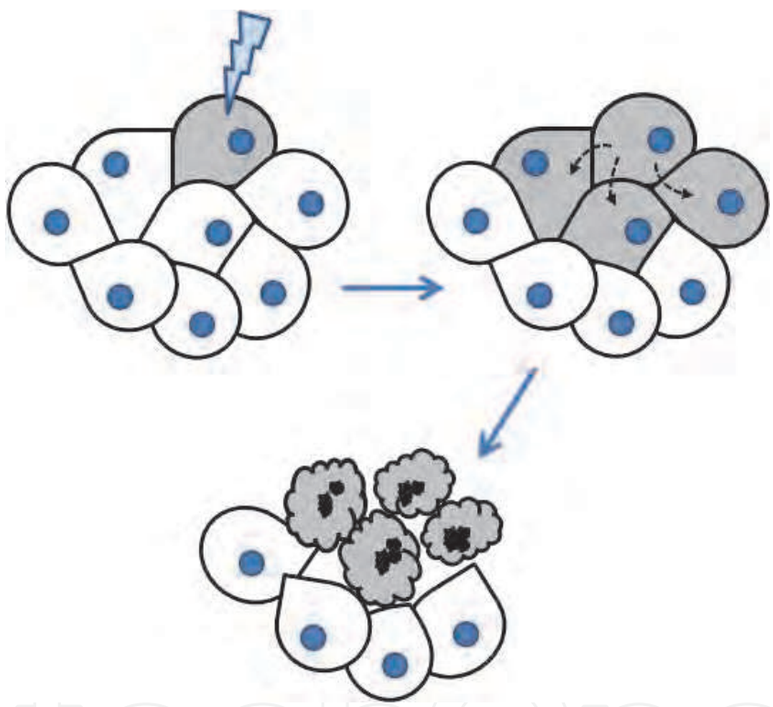

Fig. 1. Bystander Effect. A death signal, such as the one from the enzyme/prodrug system, triggered in a single cell is transmitted, through GJIC-dependent or –independent mechanisms, to neighboring cells. These bystanding cells are, in turn, destined to die without being directly targeted by the cytotoxic stimuli.

The efficacy of the enzyme/prodrug approach in vitro and in animal xenograft models has been demonstrated (Xu and McLeod 2001). However, there are many factors which immediately affect the efficacy of the approach. Although the % of cells expressing either HSVtk or CD has been shown to have some importance, the extent of contacts between cells was found to be the most crucial condition, as it requires contact between cells as well as their ability to transfer small cytotoxic molecules from one to another (Bi et al. 1993; Fick et al. 1995; Trinh et al. 1995). Using a murine breast cancer model transgenic for the activated rat neu oncogene under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR), the efficacy of the HSVtk/GCV system has been shown in vivo.

However, this approach showed an incomplete antitumor potential, the limiting factors being low viral transduction efficiency and functionality of the BE and GJIC in mammary tumor cells (Sacco et al. 1996; Sacco et al. 1995).

As previously stressed, there is need for further deciphering of the respective roles played by GJIC, Cxs and the BE in these enzyme/prodrug systems in different cellular and cancer contexts. Characterizing the interdependence of the BE and GJIC in gene therapy systems could allow their more effective use. It has been reported that the BE resulting from the thymidine kinase/ganciclovir (tk/GCV) system requires functional GJIC while in the thymidine phosphorylase/5′-deoxy-5-fluorouridine (tp/DFUR) system, whereas thymidine phosphorylase (TP) converts 5′-deoxy-5-fluorouridine (5′-DFUR, doxifluridine) to 5-FU and its anabolite 5-fluoro-2′-deoxyuridine (5-FdUrd), the BE occurs via the cell culture medium and is independent of GJIC and apoptosis. Nevertheless, combining these two systems showed more BE than each system separately (Denning and Pitts 1997). It has also been reported that, in comparison to the HSVtk/GCV system, bystander killing resulting from the CD/5-FC system is GJIC-independent, and both communication-competent and -incompetent CD-transduced cells were killed dramatically more than bystander cells (Lawrence et al. 1998). Shared culture medium rather than direct cell-cell contacts were incriminated in the BE-mediated cell killing (Bai et al. 1999). Taken together, these findings particularly support the need for a better understanding of GJIC-independent BE to better rationalize the therapeutic use of this approach. This is particularly true when combining enzyme/prodrug targeting with connexin overexpression.

2.2 Role of apoptosis in the bystander effect cytotoxicity

The cytotoxic effects of these enzyme/prodrug systems via the BE are due to the induction of apoptosis (Hamel et al. 1996). GJIC can either mediate apoptotic cell death or potentiate the efficacy of pro-apoptotic agents. The BE allows these drugs or their signaling intermediates to reach by diffusion more cells than they would do alone (Peixoto et al. 2009) (Jensen and Glazer 2004; Udawatte and Ripps 2005). In fact, it has been shown that gap junctions remain open during the apoptotic process (Cusato et al. 2006). However, there are additional, less understood mechanisms for the role of gap junctions in BE cytotoxicity. In other respect, the BE can be instrumental in drug resistance. For instance, Src activation induces Cx43 tyrosine phosphorylation and GJIC decrease, resulting in resistance to Cisplatin (Peterson-Roth et al. 2009).

Therefore, one expected limitation to the enzyme/prodrug gene therapy approach would come from the fact that in cancer cells, many apoptosis-related signaling pathways are often aberrant. It has been shown for example that HSV-tk/GCV-induced BE is influenced by mutations in p53 (van, I et al. 2005), a tumor suppressor gene frequently mutated in cancer and which regulates apoptotic cell death. A study by Garcia-Rodríguez et al. showed a strong correlation of E-cadherin expression and the TK/GCV bystander effect and that increasing the expression of E-cadherin improved TK/GCV cytotoxicity and triggered a potent antitumoral effect in vivo, through reduction of the anti-apoptotic protein Bcl-2 (Garcia-Rodriguez et al. 2011a). Similarly, the efficacy of this gene therapy strategy could be undermined by certain treatment combinations. Treatment with dexamethasone significantly reduced their apoptotic response in glioma cells, as a result of diminished GJIC-dependent BE and efficacy of HSVtk gene therapy (Robe et al. 2005). This finding warns against future usage of dexamethasone as a symptomatic treatment if HSVtk gene therapy were to be attempted. Luckily, the outcome of this gene therapy strategy can also be improved by a multitude of other treatments (Robe et al. 2004) as will be discussed below.

3. Strategies to potentiate the bystander effect-based therapy

Attempts to use the BE in gene therapy studies are limited by the ability of target cells to communicate by gap junctions. Restoring GJIC to these cells in the enzyme/prodrug systems could not only bypass this limitation, it by itself has a gene therapy potential (Figure 2). Indeed, two different approaches have been used in the literature: 1) intratumoral delivery of Cx-encoding vectors that could either be used to enhance enzyme/prodrug gene therapy or potentiate the effect of pharmacological drugs, and 2) pharmacological induction of Cx expression and GJIC, which could be combined to enzyme/prodrug gene therapy.

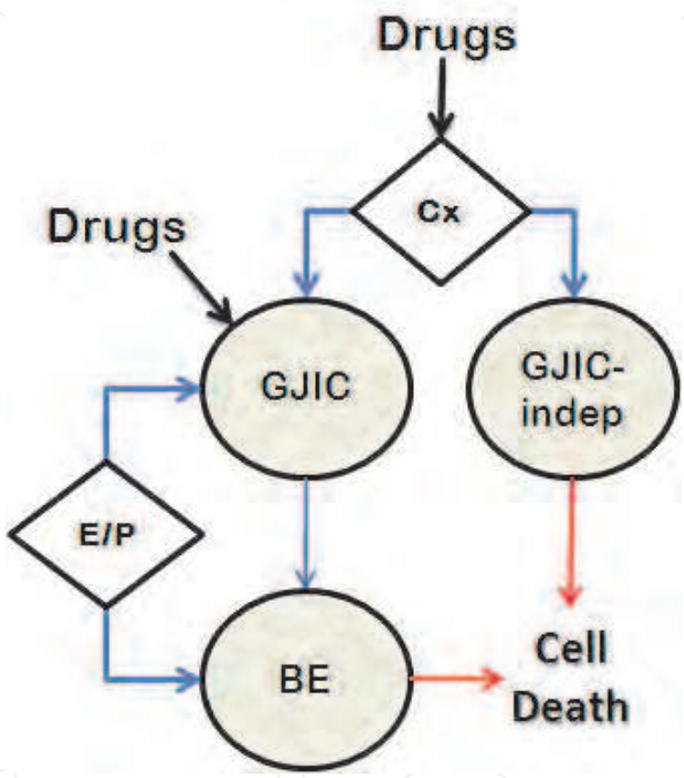

Fig. 2. Different GJIC, BE and/or Cx-based gene therapy approaches. Connexins (Cx) restoration could be performed either by direct gene delivery or by induction using pharmacological drugs. The Cx tumor suppressing effect is then either GJIC-dependent or independent (indep). Similarly, the BE-mediated cytotoxic effect of the enzyme/prodrug (E/P) approach could either be GJIC-dependent or independent. It could be improved by Cx restoration or by pharmacological intervention.

3.1 Combined enzyme/prodrug/connexin gene therapy

A major hurdle facing the enzyme/prodrug approach proved to be the loss of connexins and GJICs in the target cells, the malignant ones. Therefore, increasing the levels of Cxs and GJIC in cancer cells would result in a better response to BE-based gene therapy cytotoxicity. Transfecting cells with vectors encoding viral thymidine kinase and connexin genes has proven efficient in many studies (Cirenei et al. 1998; Ghoumari et al. 1998; Marconi et al. 2000; Tanaka et al. 2001a)(table 1).

Enzyme/Prodrug system	Connexin	Targeting Vector	Cell type	Reference
HSVtk/GCV	Cx43	Retroviral	Glioblastoma (U-87)	(Cirenei et al. 1998)
HSVtk/GCV	Cx43	Plasmid	Hepatocellular carcinoma cells (Hepa1-6)	(Ghoumari et al. 1998)
HSVtk/GCV	Cx43	A Herpes simplex viral vector (HSV)	Glioblastoma (U-87) and fibrosarcoma (L929)	(Marconi et al. 2000)
HSVtk/GCV	Cx26	Adenoviral multigenic	Bladder cancer (UM-UC-3 and UM-UC-14)	(Tanaka et al. 2001a)
HSVtk/GCV	Cx26	Retroviral	Pancreatic tumor cells (NP-9, NP-18, NP-31)	(Carrio et al. 2001)
HSVtk/GCV	Cx43	Plasmid	Cervical cancer (Hela)	(Tanaka et al. 2001c)
HSVtk/GCV	Cx43	Plasmid	Cervical cancer (Hela)	(Duflot-Dancer et al. 1998)
HSVtk/GCV	Cx43	Retroviral	Breast cancer (MDA-MB-435)	(Grignet-Debrus et al. 2000)

Table 1. Examples of gene therapy studies combining the enzyme/prodrug and Cx restoration approaches.

Nevertheless, enforced expression of Cxs might not always be sufficient to alleviate the inefficiency of the enzyme/prodrug system. For example, in a study of the efficacy of the HSVtk/GCV system combined with overexpression of Cx26 in a panel of pancreatic tumor cells, not all cell lines showed improved CJIC or bystander cytotoxicity (Carrio et al. 2001). Inability of Cx43 to properly localize at the cell surface prevented human colon tumor cells from being targeted by the BE and cytotoxicity of HSVtk (McMasters et al. 1998). The localization of Cx43 and the level of gap junctions functionality were also found to influence the BE in glioblastoma cells (Cottin et al. 2008). Therefore, a better understanding of the mechanisms involved in the stability and trafficking of connexins as well as the process of gap junction formation is needed. In particular, connexins' phosphorylation is an essential post-translational modification in their life cycle (Solan and Lampe 2009) and so are their stability and degradation by the lysosomal and proteasomal systems (Leithe and Rivedal 2007). For example, abnormal trafficking and lysosomal degradation can impede with the function of Cx43 (Qin et al. 2003a). Apigenin, a cancer chemopreventive flavonoid, was able to improve the effect of HSVtk only after concurrent transfection with the Cx43 gene, which suggests that, unlike other chemicals, it affects gap junction functionality rather than inducing connexin expression (Touraine et al. 1998). It has also been suggested that different

connexins might have different abilities to modulate the BE. Cx32 and Cx26 were reported to be significantly more effective than Cx43 at mediating the BE in cocultures of connexin-expressing and HSVtk-expressing C6 glioma cells (Jimenez et al. 2006).
Another issue that is not completely elucidated is the importance of targeting tumor cells to express both the suicide gene and the connexin at the same time. It has been suggested that separate introduction of the HSVtk and connexin genes in tumor cells might have higher killing efficiency than simultaneous expression, as illustrated by transfection of HeLa cells with Cx43 and HSVtk genes (Tanaka et al. 2001c). Interestingly, the Cx-expressing cells induce the Cx-devoid cells to contribute to GJIC through an unknown mechanism (Tanaka et al. 2001b). This is an encouraging observation in view of the known heterogeneity of tumors or in situations where Cx-negative malignant cells are scattered within Cx-positive normal tissues (i.e. gliomas), which means that gene therapy targeting of these tumors with the enzyme/prodrug system might still be efficient even when only a small subpopulation of tumor cells expresses connexins.

3.2 Combined connexins delivery and pharmacological treatments.

Modulating GJIC and Connexins has been used to sensitize to chemotherapy using a variety of pharmacological drugs (Figure 2). For example, Cx32 expression enhanced the sensitivity of human renal cell carcinoma (RCC) cells to vinblastine (VBL) *in vitro* and *in vivo* (Sato et al. 2007c). Cx43 overexpression increased the sensitivity of the LNCaP prostate cancer cells to tumor necrosis factor alpha (TNFalpha), anti-Fas antibodies, and TRAIL (Wang et al. 2007). Overexpression of Cx26 improved the growth suppressive effect of doxorubicin in prostate cancer cells (Tanaka and Grossman 2004). Restoring Cx43 expression in human glioblastoma increased sensitivity to Etoposide, Paclitaxel (Taxol) and Doxorubicin, in a way that, paradoxically, seems GJIC-independent (Huang et al. 2001). Combining intratumoral injection of a Cx43-expressing vector and intravenous injection of Docetaxel (DTX) improved anti-tumor efficiency more than DTX alone (Fukushima et al. 2007). The overexpression of Cx26 resulted in increased GJIC and enhanced cytotoxic BE of gemcitabine, a nucleoside analogue drug whose phosphorylated form is transmitted through gap junctions, in pancreatic cancer cells both in vitro and in mice (Garcia-Rodriguez et al. 2011b). At low doses, PKI-166, a Her-2/Her-1 inhibitor and PP1, a Src family inhibitor, were shown to enhance the tumor-suppressive effect of Cx32 in human renal cell carcinoma Caki-2 cells, partly through GJIC (Fujimoto et al. 2005b; Fujimoto et al. 2005a). As a last example, Cx32 expression also significantly potentiated the cytotoxicity of vinorelbine (VBN), in lung adenocarcinoma A549 cells (Sato et al. 2007a).

3.3 Combined pharmacological restoration of connexins and gene therapy

In addition to exogenous delivery of connexins, their expression can be increased using pharmacological treatments that affect different levels of gene regulation. The strategy of restoring Cx expression to favor BE-mediated cytotoxicity is mainly confronted to a flagrant misunderstanding of the mechanisms of loss of Cx expression in cancer. Many transcriptional and post-transcriptional aberrations have been described so far but, as expected, none provide a ubiquitous explanation (Carystinos et al. 2003; Gao et al. 2007; Villares et al. 2009; Leithe and Rivedal 2007; Solan and Lampe 2009). Nevertheless, the available knowledge has significantly been used in a therapeutic perspective. Transcriptional silencing of Cx expression has been shown to involve epigenetic events such as promoter methylation and chromatin acetylation. Restoration of Cx32 in human RCC

cells by 5-aza-2'-deoxycytidine (5-aza-CdR), a DNA demethylating agent, suppressed tumor growth in a xenograft model (Hagiwara et al. 2008). 4-phenylbutyrate (4-PB), a histone deacetylases inhibitor (HDACi), induced connexin expression and enhanced GJIC between pancreatic cancer cells in culture and potentiated HSVtk/GCV bystander killing effect in glioma cells (Ammerpohl et al. 2004; Ammerpohl et al. 2007). Other HDACi such as Trichostatin A (TSA) and sodium butyrate (NaBu), restored Cx43 expression and increased GJIC (Hernandez et al. 2006; Ammerpohl et al. 2007). It is not known if these HDACi would affect the outcome of the suicide gene therapy. Some of the compounds might also affect the BE independently of their gene expression-modulatory functions. N-butyrate, an inducer of histone hyperacetylation, was shown to enhance the GJIC and the BE in GJIC-deficient glioma cells independently from its HDACi function (Robe et al. 2004).

Post-transcriptional regulation of Cx expression via mRNA trafficking, stability, splicing and translation, are probably the least studied aspects of Cx life cycle and their impact in gene therapy improvement is still far-fetched. There is fortunately little more data on post-translational regulation, especially protein modification and degradation by proteasomal and lysosomal mechanisms (Kjenseth et al. 2010; Berthoud et al. 2004). Additional regulatory mechanisms include microRNA (Anderson et al. 2006) (Yang et al. 2007; Kedde et al. 2007). In addition, an active Cx43 pseudogene (PsiCx43) has been identified and found to be expressed in breast cancer cell lines but not in normal breast epithelial cells (Kandouz et al. 2004). Inhibition of this pseudogene using short interfering RNAs (siRNAs) can be used to restore Cx43 expression, thus improving chemosensitization of breast cancer cells (Bier et al. 2009). Although there are ways to specifically target these different pathways to restore Cx expression, it is not known whether this would succeed in enhancing the BE cytotoxicity.

In principle at least, connexins could also be targeted via various interaction partners that affect their localization, turnover and function such as the interaction of Cx30 with cytoskeletal (microtubules, actin filaments) and tight/adherens junction proteins (Carette et al. 2009; Qu et al. 2009) or the interaction of Cx43 with the Rab GAP-like protein CIP85 (Lan et al. 2005).

3.4 Combined pharmacological/gene therapy

Another strategy is the use of chemical inducers of Cx expression to improve the efficiency of the enzyme/prodrug gene therapy (Figure 2). The inhibition of ATP-sensitive potassium (KATP) channels with tolbutamide resulted in increased Cx43 and GJIC, enhancing the bystander effect in HSVtk/GCV therapy in U373 human glioma cells (Paino et al. 2010). All-trans retinoic acid was shown to induce Cx43 expression and to increase GJIC in tumor cell lines, resulting in an increased efficiency of the HSVtk/GCV-induced cytotoxicity in vitro and in vivo (Park et al. 1997). A similar result was observed after 8-bromo-cyclic-AMP treatment, (Carystinos et al. 1999; Kunishige et al. 1998). This approach has particularly been viewed as a chemopreventive one (King and Bertram 2005). The green tea flavonoid compound (–)Epicatechin, prevents tumor promoting chemicals such as the 12-O-tetradecanoylphorbol-13-acetate (TPA) from inhibiting GJIC (le-Agha et al. 2002). Resveratrol (3,5,4'-trihydroxy-stilbene), a natural polyphenol, provides a similar preventive effect against TPA and the insecticide DDT (Nielsen et al. 2000), and so do carotenoids (Zhang et al. 1991). It is yet to be examined whether these treatments could increase the cytotoxic potential of the HSVtk/GCV and other gene therapy systems, but we could already infer from the available data that pharmacologic upregulation of Cxs and gap junctions could be useful to combine with these gene therapy systems in clinical trials.

4. GJIC-independent and BE-independent connexin cytotoxic effects

Part of the reasons why the original strong faith in the strategy to target connexins in the treatment of human tumors has been shaken is due to the focus on the BE and GJIC only. However, it is now obvious that in many contexts, the tumor suppressor effect of Cxs' overexpression is GJIC-independent (Li et al. 2008b). Cx43 affect angiogenesis *in vitro* and *in vivo* (McLachlan et al. 2006) and improves the resistance to the chemotherapeutic agent cisplatin (CDDP) (Sato et al. 2009) in a GJIC-independent fashion. Cx26 regulates angiogenesis-related molecules by mechanisms that are both GJIC-dependent and –independent (Kalra et al. 2006; Qin et al. 2003b). GJIC-independent functions of Cx32 in blocking proliferation, invasion and metastasis in human renal cell carcinoma RCC cells, have also been reported (Sato et al. 2007b). Therefore, connexins could be used in gene therapy regardless of their ability to trigger BE or GJIC (Figure 2). The exact mechanisms and conditions where this strategy would be most effective are yet to be determined.

5. Targeting tumor cells through GJIC with their cellular environment

Another advantage of BE-based gene therapy strategies is that treatment could be aimed not only to the tumor cells but also to cellular partners within the microenvironment such as stromal and endothelial cells. For instance, when HSVtk-transduced endothelial cells and non-HSVtk-transduced tumor cells were co-cultured, treatment with GCV resulted in the BE-dependent death of both endothelial and tumor cells in vitro and in vivo (Trepel et al. 2009). Targeting human umbilical vein endothelial cells (HUVECs) with a Cx37-encoding adenovirus induced their death by apoptosis (Seul et al. 2004).

Although largely hypothetical at this point, we can envision a strategy where GJIC between tumor and stromal cells would be enforced to render tumor cells susceptible to cell killing. In other words, targeting tumor-associated endothelial cells for example, with delivery of connexins and an enzyme/prodrug system, could result in the demise of both the tumor and its irrigating blood vessels. Using a tridimensional model of cell culture, Benalalam et al. showed that GJIC between endothelial and tumor cells are required for antigenic peptide transfer to endothelial cells resulting in the latter's recognition and elimination by cytotoxic T cells (CTL) (Benlalam et al. 2009). Using the attraction of bone marrow-derived stem cells (BMSCs) for glioma cells, Huang et al. took advantage of GJIC between the two cell types to improve the efficiency of the HSVtk/GCV suicide gene therapy. Indeed, combining the expression of HSVtk by BMSCs and the expression of Cx43 by glioma cells enhanced the bystander effect and improved suicide gene therapy (Huang et al. 2009). Similarly, the formation of gap junctions between adipose-tissue derived human mesenchymal stem cells (AT-MSC) and human glioblastoma cells contributed to bystander cytotoxicity of HSVtk (Matuskova et al. 2009).

Paradoxically, GJIC-enabled bystander cells have been shown to confer protection against GCV to the very HSVtk-transduced cells that are the source of the cytotoxic signal. The impact of this observation on the therapeutic efficacy is not known. Indeed, as suggested by the authors of this study, it can either increase the efficacy of the treatment, by decreasing the demise of the HSVtk cells, thus prolonging their cytotoxic effect, or have an opposite effect by increasing their survival (Wygoda et al. 1997). Nevertheless, this observation shows that the so-called "bystander cells" and their ability to communicate by GJIC are an important element to take into consideration in the BE-based therapy. This applies to tumor cells as well as the stromal cells with which they communicate.

The therapeutic potential of the neural stem cells (NSCs) in the treatment of brain tumors have been demonstrated and, in many reports, have been shown to rely on bystander effect. NSCs are highly migrating cells able to cross the blood–brain barrier and which show tropism for tumor cells. Many studies showed that NSCs can be genetically modified to target tumor cells and the use of the cytosine deaminase (CD)/5-fluorocytosine (5-FC) system delivered particularly important results in medulloblastomas and gliomas (Aboody et al. 2000; Shimato et al. 2007; Kim et al. 2006) as well as breast or melanoma metastases to the brain (Joo et al. 2009; Aboody et al. 2006). Combined delivery of the CD/5-FC system with Interferon-β (IFN-β), known for its anti-tumor effects, showed a stronger bystander killing effect in glioma both in vitro and using an orthotopic xenograft in vivo model, where animals were intravenously infused with CD/IFN-β-expressing NSCs and administred with the prodrug 5-FC (Ito et al. 2010). Also, migratory HSVtk-transduced NSCs were able to kill untransduced glioma cells by a GJIC-mediated BE (Uhl et al. 2005).

An additional level of complexity is the formation of different types of GJICs between different cell types. Homotypic gap junction channels formed of identical connexons and heterotypic channels made of connexons containing different connexins (Vaney and Weiler 2000; Kapoor et al. 2004) can show different permeabilities (Weber et al. 2004; Bevans et al. 1998b). This implies that Cx-mediated gene therapy will necessitate an elaborate "customization" effort to target specific interactions and avoid non specific effects. For instance, transformed cells form GJICs between them that are independent of the GJICs formed within adjacent nontransformed cells, with apparently no heterologous communication (Yamasaki and Katoh 1988). Therefore, it is in principle possible to target cancer-specific GJIC compartments without affecting normal cells.

6. Clinical trials

Many clinical trials have been performed to validate the enzyme/prodrug gene therapy approach and test its effects. These include the trial of adenovirus mediated delivery of HSVtk combined with GCV treatment in operable primary or recurrent high-grade gliomas, which resulted in a clinically and statistically significant increase in mean patient survival (Immonen et al. 2004). A phase I dose escalation clinical trial was conducted in 11 men with localized recurrent and metastatic hormone-refractory prostate cancer. In this trial, an adenovirus vector carrying osteocalcin promoter-driven HSVtk was used to target both prostate cancer cells and their neighboring stromal cells and valacyclovir, another anti-herpetic prodrug converted to aciclovir, was given orally. The results showed a good tolerance with no serious adverse events but with local cell death in treated lesions in 63.6% of patients (Kubo et al. 2003). In another clinical phase I/II study, 36 prostate cancer patients with local recurrence after radiotherapy which received single or repeated cycles of adenoviral vector-mediated HSVtk/GCV intraprostatic gene therapy (Miles et al. 2001) showed no significant side effects and a significant increase in biological responses such as the mean serum PSA-doubling time (PSADT), prostate-specific antigen recurrence (PSAR), return to initial PSA (TR-PSA), and activated CD8(+) T cells present in the peripheral blood. In another phase I trial, nine courses of intraprostatic injections of adenoviral HSVtk followed by intravenous injection of GCV in 8 patients with local recurrence of prostate cancer after definitive hormonal therapy, showed no adverse events and a significant prolongation of the median serum PSADT. In five patients, decrease of PSA values was also observed (Nasu et al. 2007). Also, intraperitoneal administration of an HSVtk-encoding

adenovirus and intravenous GCV showed significant efficacy in women with recurrent ovarian cancer (Alvarez et al. 2000). Finally, an adenoviral vector encoding the HSVtk gene was also used in a phase I trial where it has been introduced into the pleural cavity of patients with malignant mesothelioma in combination with systemic GCV treatments and showed good tolerance and detectable gene delivery (Sterman et al. 1998).

Most of these clinical trials focused on issues of tolerance of the therapy and efficacy of delivery. Although it is understood that these gene therapy attempts rely mainly on the BE, it is frustrating that no data is available that correlates these clinical results with the formation of gap junctions and Cx expression in the targeted tumors.

7. Conclusion

Bystander effect is a big step forward in attempts to use gene therapy in cancer treatment. The idea that one can kill more cells than initially targeted has been a revolutionary concept. However, the biggest challenge to the translation of this concept into an effective therapy has been the lack of information in many aspects surrounding the BE and the role of GJIC and connexins. As further basic science studies are performed, we will be able to comprehend the mechanisms of loss of Cx expression in cancer and how they could be alleviated. Whether and how these mechanisms could be used to improve gene therapy is, again, wide open to exploration. An immediate impact of these studies would be the analysis of tumors for GJIC or Cx expression to identify a subset of patients most likely to benefit from gene therapy using enzyme/prodrug systems such as HSVtk/GCV.

Other potential strategies could make use of the ability of gap junctions to transmit different types of cytotoxic signals. Radiotherapy for instance could benefit from this knowledge, based on the finding that death signals could be transmitted through BE from irradiated to nonirradiated cells (Azzam et al. 2001; Prise and O'Sullivan 2009). Radiation therapy could be combined with gene therapy interventions aimed at increasing GJIC which would amplify the cellular responsiveness to radiation therapy

The function of gap junction channels involves conductance and electrical and chemical gating that can be affected by many factors, including the nature and stoichiometry of the contributing connexins, ensuring selective permeability to various molecules (Saez et al. 2010; Nakagawa et al. 2010; Bevans et al. 1998a). In theory, BE-based gene therapy combined with Cx expression restoration would benefit from identifying Cxs with the best conductance in specific cancer settings. Furthermore, it would be possible to introduce specific mutations that would improve the conductance of BE cytotoxic molecules. Another possibility is to regulate opening and closure of gap junctional channels. The search for chemical inhibitors has delivered a series of drugs that result in either opening or closure of gap junctions (Salameh and Dhein 2005). Another approach involved the use of mimetic peptides that bind to connexin hemichannels, and modify their conductance (Evans and Leybaert 2007). Although it is yet to be assessed, targeting GJ opening and conductance properties could optimize the effect of the BE gene therapy. However, again, the relevance to cancer of channel gating functions of connexins versus GJIC-independent functions is a major unknown. Although this pharmacological approach is most likely to be successful in diseases such as arrhythmia or seizure, where hyperpolarization is a major issue, its possible impact in cancer therapy, especially in combination with gene therapy, should not be excluded.

Another issue of potential importance in improving the efficiency of the BE-based gene therapy is the nature of metabolites that could or could not be transmitted by BE as

illustrated by the differential ability of pyrimidine nucleoside analogues such as GCV to pass the gap junctions and trigger cytotoxicity (Degreve et al. 1999). Enzyme/prodrug combinations involving pyrimidine analogues (BVDU, BVaraU) presented smaller bystander killing than the combination involving the purine analogue (GCV) (Grignet-Debrus et al. 2000).

In addition to connexins, another family of proteins called pannexins is increasingly being scrutinized for their ability to form gap junctions (D'hondt et al. 2009). Unless and until their role in cancer and GJIC has been clearly established, the only GJIC-based therapeutic strategies will likely keep focusing on connexins.

Finally, so far only clinical trials on localized malignancies have been conducted, such as direct intra-tumoral injection of the vector in glioma therapy. Future studies are necessary to develop intravenous delivery of viral vectors in the enzyme/prodrug gene therapy approach, to allow targeting of other cancers. In addition, these Phase I trials have mainly addressed safety, toxicity and gene delivery issues. Further assessment of the anti-tumor effects and the correlation with GJIC and connexin expression should absolutely be on the list of future clinical trials. Combinations of these gene therapy approaches with other cancer therapeutic modalities should also be considered.

In summary, the promises of the Bystander effect, GJIC and Connexin-based gene therapies are still alive. It is possible that the great enthusiasm for their potential was so high that it blinded us to the urgency of further examination of their mechanisms and regulations which, once performed, would much significantly improve the rationalization of the clinical application and outcome.

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

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