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Cancer Gene Therapy: Combination with Radiation Therapy and the Role of Bystander Cell Killing in the Anti-tumor Effect

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Current anti-cancer modalities such as surgery, chemo- and radiation therapies have only limited success in cancer treatment. Gene therapy is a promising new tool to improve outcomes. In this review, first we summarize the various strategies to kill tumor cells, and then focus on the bystander effect of gene therapy. A variety of strategies, such as gene-directed enzyme pro-drug therapy, activation of an anti-tumor immune attack, application of replication-competent and oncolytic viral vectors, tumor-specific as well as radiation- and hypoxia-induced gene expression, might be applied to target tumor cells. We put special emphasis on the combination of these approaches with local tumor irradiation. Using the available vector systems, only a small portion of cancer cells contains the therapeutic genes under clinical situations. However, cells directly targeted by gene therapy will transfer death signals to neighboring cancer cells. This bystander cell killing improves the efficiency of cancer gene therapy. Death signals are delivered by cell-to-cell communication through gap junction intercellular contacts, release of toxic metabolites into the neighborhood or to larger distances, phagocytosis of apoptotic bodies, and the activation of the immune system. Bystander cell killing can be enhanced by the introduction of gap junction proteins into cells, by further activating the immune system with immune-stimulatory molecules, or by introducing genes that help the transfer of cytotoxic genes and/or metabolites into bystander cells. In conclusion, although bystander cell killing can improve therapeutic effects, there should be additional developments in cancer gene therapy for a more efficient clinical application. (Pathology Oncology Research Vol 12, No 2, 118–124)

Key words: gene therapy, bystander effect, gap junction

Introduction

Gene therapy is a potential candidate to improve survival rates in cancer patients. So far, however, the ongoing clinical trials have not presented many promising data. One possible explanation for the unconvincing results is that the first generational viral vectors can penetrate only a small portion of the tumor cells, which is not sufficient for tumor cure. Because of the low penetration capability, the bystander effect is an absolute requirement to the future success of cancer gene therapy. As stated by Vile et al., "No single gene can be a serious contender, unless it has a demonstrable bystander effect".[1]

In this review, we summarize the various basic gene therapy protocols, and focus on the bystander effects, which might improve the anti-cancer potential.

Basic gene therapy strategies and combinations with radiation therapy

Suicide genes in gene-directed enzyme pro-drug therapy

Gene-directed enzyme pro-drug therapy (GDEPT) with drug-sensitizing genes is a promising tool to overcome resistance and to decrease the unfavorable side effects of chemotherapy.[2] In GDEPT, tumor cells are transduced with suicide genes that can convert non- or mildly toxic drugs to highly toxic metabolites. The most frequently

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used GDEPT protocol is the thymidine kinase/ganciclovir system. The *Herpes simplex*-derived thymidine kinase (TK) converts ganciclovir (GC) to ganciclovir-monophosphate, which is further phosphorylated by cellular kinases to toxic ganciclovir-triphosphate. Mammalian cells lack TK, thus GC causes toxic effects only in cells transfected with TK.[3-5]

A widely applied cancer chemotherapy agent is 5-fluorouracil (5-FU). In mammalian cells, 5-FU is metabolized first into nucleoside fluorouridine by uridine phosphorylase and then phosphorylated into 5-fluoro-2'-uridine-5'-monophosphate (FUMP) by uridine kinase.[6] Unfortunately, 5-FU resistance and toxic side effects are frequent in cancer patients.

There might be two possibilities to overcome this problem. One of them is to produce 5-FU from the non-toxic 5-fluorocytosine (5-FC) by bacterial or yeast cytosine deaminase enzymes (CD) through GDEPT.[3,4] Another possibility is to introduce the *E. coli* uracil phosphoribosyltransferase (UPRT) gene into the tumor cells, which converts 5-FU directly and very efficiently into FUMP.[2,7]

The authors of this review used a double-suicide GDEPT system against murine brain tumors.[8] The applied adenoviral vector encoded both the TK and the UPRT genes. Intratumor injection of this vector and subsequent treatment with the corresponding agents substantially slowed down tumor progression. They have found that under *in vitro* conditions, the combination of 5-FU and ganciclovir treatments with irradiation increased cytotoxicity by three orders of magnitude. In glioma-bearing mice, the combined GDEPT and radiation treatment slowed down tumor progression and improved survival rates.

Activation of the anti-tumor immune response

There are several immunotherapeutic approaches that might increase the immunogenicity of the tumors. One possibility is the introduction of cytokine-encoding genes into the tumor cells. It is expected that the host immune system is activated against the tumor, and will attack the cancer cells present at the primary tumor site and at distant metastases.[9-11]

Several reports,[12,13] including ours,[14] suggested that the combination of radiation therapy with intra-tumor administration of a cytokine-encoding vector or with vaccination with cytokine-secreting autologous cancer cell vaccines substantially slowed down tumor progression. One simple explanation for the synergistic effect of vaccination and radiation therapies is that there is a continuous competition between tumor growth and tumor eradication by the activated immune system. Local irradiation decreases the tumor burden, so the activated immune system could overcome the decreased tumor mass.

Replication-competent and oncolytic viruses

After intra-tumor delivery of the first-generational viral vectors, the infection is limited to cells surrounding the needle track. The low penetration ability might be overcome by viral vectors, suitable to propagate in tumor cells. Some of the replicative vectors have oncolytic capacities, as well. One of the first conditionally replicative, oncolytic vectors was the ONYX-015 adenovirus.[15] In ONYX-015 the E1B region was removed from the wild-type adenovirus. The E1B protein has two different roles in infected cells. It helps turning on the expression of late adenoviral genes, and binds to and inactivates the cellular p53 protein. In the absence of E1B, p53 inhibits adenovirus replication in normal cells. Because p53 is absent or mutated in most of the cancer cells, the ONYX virus might replicate in and kill the p53-deficient tumor cells. The anticancer effect of ONYX-015 is under evaluation in a few clinical trials including head and neck cancer and metastatic lung tumors.[16,17] ONYX is much more effective when combined with radiation in colon carcinoma and glioma tumor models.[18,19] Some viruses, such as vaccinia, measles, herpes simplex, Newcastle disease virus can preferentially replicate in tumor cells and demonstrate oncolytic activities.[20] Ionizing radiation improves the oncolytic effect of herpes simplex[21], vaccinia[22] and Newcastle disease (Sáfrány et al., manuscript in preparation) viruses.

Tumor-specific and radiation-driven therapeutic gene expression

In cancer gene therapy it would be highly preferable if the therapeutic genes were expressed and/or the vectors replicated only in the targeted tumor cells. To achieve this, gene expression and/or vector replication should be placed under the control of tumor-, radiation- or hypoxia-specific promoters.[23-26.] The EGR1 radiation-induced promoter contains four copies of the $CCAT_6GG$ sequence (CArG element), which is responsible for radiation induction (3-fold by 2 Gy).[27,28] Several viral vectors were constructed where the expression of the therapeutic gene was placed under the control CArG elements.[29,30] When breast cancer, lung, rectum, pancreas tumor and melanoma patients were treated with the vector and tumor irradiation, very promising results were obtained.[29]

The $p21^{WAF1}$ promoter is also induced by radiation.[23] When the inducible nitric-oxide-synthase (iNOS) gene was placed under the control of the WAF1 promoter, significant tumor growth delay, apoptosis induction and tumor cell radiosensitization were achieved.[31,32]

Hypoxia-induced gene expression

Tumor hypoxia is usually associated with aggressive disease and poor prognosis. Tumor hypoxia might be utilized in cancer gene therapy by putting the therapeutic genes

under the control of hypoxia-responsible elements (HREs). HREs are enhancers containing the (A/G)CGT(G/C)(G/C) sequence and are present in the promoter region of several hypoxia-responsive genes, such as vascular endothelial growth factor (VEGF), erythropoietin and phosphoglycerate kinase.[23,33] When five copies of HRE were linked to a minimal CMV promoter, hypoxia induced a 500-fold gene expression.[34]

Clinical trials

By January 2006, at least 1132 gene therapy clinical trials have been initiated, most of them in the USA (742) and Europe (327). Sixty-seven percent of these trials aim to cure cancer. So far, only few of them have reached phase III, many of them did not get beyond phase I.[35] Most of the anticancer trials applied the TK-GC protocol. One of the biggest, randomized phase III trial was conducted against glioblastoma multiforme (GBM).[36] Two-hundred-forty-eight patients were treated either with standard therapy (surgery + radiation) or with the combination of standard and gene therapies. In the combined gene therapy protocol the tumor site was infiltrated immediately after surgery with allogeneic fibroblasts producing a TK-encoding retroviral vector and consequently the patients were treated with GC.

The trial proved the safety of this approach, but neither disease progression nor overall survival was significantly different between the two patient groups. A similar trial, which included fewer patients (37 TK-GC treated versus 19 standard treatments), was performed in Finland.[37] The main difference was that in the Finnish trial a TK-encoding adenoviral vector was used. In this case, the gene therapy-treated group presented significantly improved mean survival rates. Again, no serious adverse effects have been observed.

The current state of anti-cancer clinical trials has been extensively reviewed.[38-41] The major conclusion is that the application of various viral vectors is safe, but so far the clinical advantage of the various protocols has not been proved. The unsatisfactory clinical results might be explained by the low tumor infiltration capability of the currently available vector systems. Despite of a significant bystander effect, transgene expression remained insufficient under clinical situations. A potential solution might be the application of replicative, oncolytic viruses. Perhaps the most prominent representative of the conditionally replicating viral vectors is ONYX-015, as mentioned earlier. The therapeutic efficacy of ONYX-015 is under evaluation in head and neck,[16] hepatobiliary[42] and brain tumors.[43] Unfortunately, a significant benefit have not been detected so far from ONYX treatments alone. However, its combination with chemotherapy is a promising approach in head and neck cancer.[44]

Bystander effects in gene therapy

It is well known that ionizing radiation has serious consequences on cells directly hit by radiation (cell death, carcinogenic mutations, genomic instability, etc.). Beside this, radiation-induced effects might be observed on cells directly not targeted by radiation. This phenomenon is called the bystander effect of radiation. The bystander effect can contribute to the death of the neighboring, directly non-targeted cells or to the development of mutations. When cancer gene therapy is combined with radiation therapy, radiation-induced lethal bystander effects might increase the death of malignant cells. In an analogous manner, genetically modified cells during cancer gene therapy may also deliver various signals to the neighboring cells. In the following chapters, we will focus on the bystander death signals that may contribute to a more efficient cancer cure.

As mentioned above, the most frequently studied gene therapeutic strategy is the TK-GC system. Ganciclovir is not toxic for mammalian cells. After initial phosphorylation by TK, cellular kinases will generate the toxic triphosphate form of GC, which kills TK-containing cells. The question is whether TK-minus cells could be killed by the bystander effects. This presumed bystander effect might present death signals or toxic pro-drug metabolites to the neighboring cells, and even to cells at distant metastases. The bystander effect might occur via intercellular communications, by phagocytosis of apoptotic bodies, through the activation of the immune system, or by the release of cytotoxic metabolites.[45,46]

The mechanisms of the bystander effects

Exchange of toxic metabolites through gap junctions

The bystander effect, produced by ganciclovir-mediated killing of cells transduced with the TK gene, defines the cooperative killing of non-transduced cells. The major contributor to this phenomenon is a metabolic cooperation involving the transfer of cytotoxic small molecules between cells mainly through cell-to-cell interactions. When TK-positive cells were co-cultured with TK-negative cells at high densities, both TK+ and TK- cells were killed by GC. However, when the cells were co-cultured at low cell densities, only the TK+ cells were killed. This suggests that cell-to-cell contact is necessary for the bystander effect and cells might communicate through gap junctions.[45,46]

Gap junctions are important mediators of direct intercellular communications. Ions, small metabolite molecules, second messengers and certain dyes can pass through gap junctions. Gap junctions consist of two hexameric integral membrane protein hemi-channels termed connexons, which interact across the narrow extracellular space to cre-

ate a complete channel. The connexons are composed of six connexin protein subunits that surround the central pore. At least 14 different connexins have been identified in mammals. Gap junctions allow the passage of molecules less than 1 kDa in size, such as triphosphorylated GC. Protein kinase A activated by cAMP-mediated signals is the only well-characterized signal transduction system that increases gap junctional intercellular communication (GJIC) in most cell types.[45,46]

It was suggested that the presence of gap junctions in the target cells is much more important than that in the effector cells.[47] Connexin expression in rat glioma 9L cells is much higher than in C6 cells. Both 9L and C6 cells were transduced with TK gene and different combinations of TK+ and TK- cells were treated with GC. A strong bystander effect was detected in 9L cells, which was absent in C6 cells. When wild-type 9L cells were mixed with TK-containing C6 cells, also a strong bystander effect was detectable. However, the bystander effect was not detectable in the mixture of wild-type C6 and TK+ 9L cells. Similar *in vivo* effects were observed when different combinations of TK+ and TK- cells were transplanted into athymic nude mice.

Further confirming the importance of target cells, C6 cells were transduced with the connexin 43 gene and mixed with TK+ C6 cells. This combination exhibited a strong bystander effect under *in vitro* conditions compared to connexin non-transduced cells.[47]

The intracellular TK level might also influence the bystander effects. Cells were transduced with either one or two copies of TK. The efficiency of GC killing and the magnitude of the bystander effect were compared for the single- and double-copy TK+ cell lines. Cells that expressed two copies of TK metabolized GC more efficiently than single-copy TK+ cells. They were also more sensitive to GC, and demonstrated improved bystander killing.[48]

Release of soluble factors

Some of the published data suggest that the presence of gap junctions is not obligatory for the bystander effects. In several instances bystander cell killing was reported when the TK+ effector and the TK- target cells were not in contact or when they were separated physically by permeable membranes, or even when the medium was transferred from one cell culture dish to the other. Princen et al. analyzed the mechanisms of the bystander effect in two cell lines showing differences in cellular communication (DHD/K12 and 9L). 9L cells exhibited a strong bystander effect, while DHD/K12 cells demonstrated only a moderate one. Chemical inhibition of gap junctions blocked the bystander effect only in 9L cells.

The transfer of culture medium from GC-treated TK+ DHD/K12 cells to untreated TK- cells induced cell death in the untreated cells, suggesting the release of toxic GC metabolites into the medium by TK-transduced cells.[49] Moreover, SW620 human colon carcinoma cells could form only a limited number of gap junctions, still they could present strong bystander signals to neighboring cells. These cells could also release toxic GC metabolites into the medium.[46,50]

It seems that the contact-independent bystander effect is cell type-dependent. Several cell lines (DHD/K12, SW620 or A15A5 rat glioma) are capable for the release of cytotoxic metabolites (the phosphorylated forms of GC) into the medium, while others (9L rat glioma) are not.[46]

Uptake of apoptotic vesicles

Some data suggest that the phagocytosis of apoptotic bodies might contribute to the bystander cell killing. After GC-treatment, TK+ cells will die mainly by apoptosis. During apoptotic cell death, apoptotic bodies are formed by the dying cells and these bodies might be phagocytosed by other, TK- cells. In this manner, TK- cells can pick up death signals that can lead to apoptotic death. The bystander effect was eliminated when apoptotic vesicle transfer was prevented.[51] However, according to other data it is also possible that toxic metabolites were already transferred to the TK- cells before phagocytosis of the apoptotic bodies, and this led to the cell death. Hamel et al. detected apoptosis in bystander cells and found that bystander cell death could be inhibited by the overexpression of the anti-apoptotic Bcl2 gene. They also proved that bystander cell death occurred before the phagocytosis of apoptotic bodies.[52]

Induction of immune responses

The immune system might have substantial contribution to bystander cell killing under *in vivo* conditions. When animals with TK+ tumors were treated with GC, the residual tumors were infiltrated by inflammatory cells. The inflammatory cells consisted of $CD4^+$ and $CD8^+$ lymphocytes, NK cells and macrophages. When tumor cells were re-injected in the surviving animals, they were rejected, demonstrating long-term immunity.[53]

Bi et al. investigated the bystander effect in an oral squamous cell carcinoma cell line growing in nude mice.[54] They transplanted the mixture of TK+ and TK- cells on one flank of the mice and TK- cells on the other flank, and treated the animals with GC. Interestingly, anti-tumor effect was observed at both tumor locations. Although nude mice are T-cell deficient, still they have intact monocytes and macrophages, and are able to produce antibodies. When this experiment was repeated in SCID-Beige mice, which are deficient in T-, B- and NK cells, but still possess macrophage activity, the anti-tumor response was

absent in the TK- tumor. The data suggest that an immune-related anti-tumor attack is responsible for the distant bystander effect.[54]

Increasing bystander cell killing potential

As summarized above, bystander cell killing contributes to the efficacy of cancer gene therapy. Improvements in bystander cell killing might further increase the anti-tumor effect of gene therapy. Several possibilities are outlined below.

Restoration of gap junctional intercellular communications

Gap junctional intercellular communications (GJIC) are very important, cell type-dependent mediators of bystander effects.[45-48] The gap junction-dependent diffusion of phosphorylated ganciclovir metabolites from transfected cells to their neighbors was proved to enhance the overall benefit of the TK-GC system. Unfortunately, tumor cells are often gap junction-deficient.[46] There are several possibilities to improve GJIC. For instance, all trans-retinoic acid can increase connexin 43 expression in various tumor cell lines and facilitate GC-induced bystander cell killing both under *in vitro* and *in vivo* conditions.[55]

Robe et al. demonstrated that dibutyryl adenosine 3',5'-cyclic monophosphate (cAMP) can induce GJIC in glioblastoma cells and improve the efficacy of TK-GC treatment.[56] In a human choriocarcinoma cell line 8-bromo-cAMP increased connexin 40 mRNA expression, gap junctional intercellular communication and the bystander effect of the TK-GC system.[57]

GJIC can also be restored by transfection of the cells with genes encoding connexin. HeLa cells are deficient in gap junctions and do not exhibit bystander cell killing by TK-GC. The introduction of the connexin 43 gene into the cells resulted in the killing of TK- cells when they were in contact with TK+ ones. This cell killing effect was absent when TK+ and TK- cells were co-cultured without direct cell-cell contact.[58] The introduction of the connexin 43 gene into the cells improved cell-to-cell communications under *in vivo* conditions, as well. When the mixture of TK+ and TK- HeLa cells were transplanted into nude mice, GC treatment had only moderate effect on tumor growth. However, when cells were transfected with the connexin 43 gene before transplantation, tumor growth retardation was highly improved after GC treatment.[59]

The effect of connexin 43 expression on the susceptibility of CNS1 and C6 rat glioma cell lines to TK-GC was investigated by Sanson et al.[60] It was found that the bystander effect in these cells correlated with gap junctional communication dependent on connexin 43 level. Transfection of C6 cells (deficient in GJIC) with the connexin 43 gene increased GJIC and bystander cell killing when the cells were in contact.[60]

Augmenting the immune-related anticancer response

Increasing the anti-tumor immune response might enhance the bystander effect as well. Walling et al. used retroviral vectors to introduce the TK and interleukin-2 genes into human osteosarcoma cells.[61] They detected a strong bystander effect both under *in vitro* and *in vivo* conditions, when the mixture of TK+ and TK- cells was transplanted into nude mice. In a second set of experiments, they transplanted two tumors into the mice. The first tumor contained only TK- cells, while the other was a mixture of TK+ and TK- cells. GC treatment caused the regression of both tumors. Growth retardation of the TK- tumor was further improved if the other tumor carried the interleukine-2 gene, beside TK, suggesting a potential role for the immune system in the distant bystander effect.

Linking the thymidine kinase gene to other proteins

It is possible to induce a gap junction-independent bystander cytotoxic effect by linking the TK gene to the gene of another herpes virus protein, VP22. The VP22 protein has been shown to pass freely between cells by an unknown mechanism. VP22 is exported from the producer cells by a Golgi-independent mechanism. VP22 has a unique ability to re-enter surrounding cells. It can spread to almost every cell in a monolayer from only a few producer cells. VP22 fusion proteins might function as potent protein delivery systems.[46] A VP22-TK construct was tested on different tumor cell lines *in vitro* and *in vivo* to improve bystander killing. The VP22-TK chimeric proteins spread between cells in sufficient quantities to induce cell death in response to GC treatment, not only in the primary TK+ cells but also in surrounding TK- cells. This effect was observed *in vitro* after GC treatment of transfected tissue culture cells, and *in vivo* after GC treatment of mice injected with tumor cells transduced with VP22-TK fusion genes. This suggests a new strategy to increase the effectiveness of suicide gene therapy for the treatment of cancers.[62]

Apoptosis-inducing therapeutic genes

Induction of apoptosis in cancer cells can be achieved by the introduction of pro-apoptotic genes (FasL, TRAIL). Fas ligand (FasL) is a membrane protein that belongs to the TNF family. It binds to the Fas receptor and induces apoptosis in sensitive cells. It was demonstrated that the introduction of FasL into prostate cancer cells by adenoviral vectors initiated apoptosis and the formation of apoptotic bodies. These apoptotic bodies were released into the local environment and phagocytosed by neighboring cells, leading to bystander cell killing.[63]

TNF-related apoptosis-inducing ligand (TRAIL) is another member of the TNF family. TRAIL induces apoptosis in

transformed, but not in normal cells. TRAIL was cloned into an adenoviral vector and transduced into cancer cell lines. Overexpression of TRAIL induced apoptosis in transduced cells and TRAIL was released into the medium. When the TRAIL-containing medium was transferred to soluble TRAIL-sensitive cell lines, it induced bystander cell death.[64]

Interferon-gamma (IFN-γ) can modulate the anticancer activities of TNF family members including TRAIL. Park et al. demonstrated that pre-treatment of cancer cell lines with IFN-γ increased the production of interferon regulatory factor-1 (IRF-1) within the cells. IRF-1 induction improved TRAIL-induced apoptosis.[65] These data suggest that TRAIL-related bystander effects might be augmented by IFN-γ treatment.

Conclusion

Animal experiments provided an enormous amount of data that cancer gene therapy might be an efficient new therapeutic agent. Despite of this fact, the ongoing clinical trials proved only the safety of these treatment modalities, but they had not contributed significantly to the survival of cancer patients. The development of new vector systems and improvements in modulating the bystander effects may give new, additional opportunities to a more successful clinical approach.

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