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Review

The Role of the Bystander Effect in Suicide Gene Therapy

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INTRODUCTION

IN RECENT years, a combination of cytogenetic and molecular techniques has allowed the identification of the precise genetic alterations that underlie the stepwise progression to malignancy. This is particularly true of colorectal carcinoma, and the genetic events associated with key stages in the clonal progression, from normal colonic mucosa through early and late adenomatous changes to localised malignancy, have been clearly identified [1–3]. Attempts have been made to exploit this knowledge to develop new therapeutic strategies for the treatment of patients with cancer, and a number of experimental approaches to 'cancer gene therapy' have been described. These include strategies designed to suppress the expression of an oncogene or to restore the function of a defective tumour suppressor gene. Alternative approaches involve either strategies designed to enhance an antitumour immune response or the insertion of genes conferring drug resistance or drug sensitivity.

The identification and precise sequencing of oncogenes has led to the development of antisense strategies for cancer gene therapy. These therapies aim to suppress the expression of a specific oncogene. Antisense oligonucleotides are short sequences of RNA that are complementary to cellular mRNA oncogene transcripts. Antisense transcripts bind to oncogene mRNA and thereby inhibit mRNA translation. In several *in vitro* models, this has resulted in the inhibition of proliferation and reversal of the malignant phenotype [4, 5]. Mutations of tumour suppressor genes also contribute to the development of malignancy. Indeed, mutation of the tumour suppressor gene *TP53* is the most common genetic alteration in human malignancy [6–8]. One of the prime functions of wild-type *TP53* is thought to be the prevention of DNA damage accumulation and chromosomal instability that may predispose tumour formation. A further approach to cancer gene therapy, therefore, involves the restoration of a defective tumour suppressor gene. *In vitro*, the transduction of wild-type *TP53* has been shown to arrest the growth of a colorectal carcinoma cell line [9] and *in vivo*, wild-type *TP53* has prevented tumour growth and led to the regression of established tumours [10].

However, there are a number of problems associated with these therapies. Perhaps the most obvious is that it may be necessary to genetically modify each individual malignant cell within a tumour in order to achieve a therapeutic effect. This is an unrealistic prospect given the efficiency of current methods of gene delivery. In addition, these strategies target only one of several genes responsible for the development of the malignant phenotype, and this is against a background of considerable genetic heterogeneity even within the same tumour type.

An alternative approach to cancer gene therapy involves strategies designed to enhance an antitumour immune response. Tumour cells may be genetically modified *in vitro* to express cytokine genes [11], MHC genes [12] or genes that encode for co-stimulatory molecules such as B7 [13]. The administration of these gene-modified cells to syngeneic immunocompetent animals has resulted in tumour rejection and the generation of a tumour specific antitumour immune response. These therapies appear to have promise as 'cancer vaccines', but this is likely to be in the context of adjuvant therapy rather than for the treatment of bulk disease.

The remaining approaches to cancer gene therapy involve the insertion of genes conferring either drug resistance or drug sensitivity. Conventional chemotherapeutic regimes are often limited by systemic toxicity and myelosuppression. The transfection of the human multi-drug resistance (MDR) gene into bone marrow results in protection from the myelosuppression induced by a number of chemotherapeutic agents [14 and refs therein]. Thus, enhanced resistance to myelosuppression may lead to the introduction of more aggressive and more effective chemotherapy regimes.

Conversely, it is also possible to modify tumour cells so that they become sensitive to an agent that is otherwise non-toxic. This involves the insertion of a gene coding for an enzyme that converts a non-toxic prodrug into a lethal compound; administration of the prodrug results in the death of the recipient cell [15]. This approach is known as suicide gene therapy. Suicide gene therapy is an attractive form of cancer gene therapy as the administration of the prodrug results not only in the death of the recipient cell, but also in the death of surrounding cells. Effective therapy

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can, therefore, be achieved without the need to introduce a functional suicide or prodrug activating gene into each individual tumour cell. Indeed, a number of studies using suicide genes have shown that it is possible to achieve complete tumour regression even though only a fraction of the tumour mass is genetically modified [16–21]. This phenomenon of the death of neighbouring untransduced cells is now known as the 'bystander effect' [17]. This review describes the development of suicide gene therapy, the discovery of the bystander effect and the evolution of our understanding of the possible mechanisms involved. The way in which the bystander effect may be enhanced and the implications that this may have for the development of future therapies are also discussed.

GENE DELIVERY SYSTEMS

Successful gene therapy for cancer will require effective methods of gene delivery. *In vivo*, this is usually achieved by means of retroviral or adenoviral vector systems, each of which has relative advantages and disadvantages. Other viral methods for gene delivery are also under development as are non-viral methods of gene delivery.

Retroviral vectors

Retroviruses are RNA viruses of approximately 10 kilobases in length. They are composed of three structural genes, the *gag*, *pol* and *env* genes. These code for viral core proteins, viral enzymes including reverse transcriptase and envelope proteins, respectively. The structural genes are flanked by long terminal repeat promoter sequences. Viral envelope proteins bind to specific cell surface receptors, this results in cell fusion and the entry into the cell of a nuclear capsid containing retroviral RNA. Within the cytoplasm, single-stranded RNA undergoes reverse transcription resulting in the formation of a single-stranded DNA template. This DNA template is then used to form double-stranded DNA which is transported to the nucleus and integrated into host chromosomal DNA.

Retroviral vectors consist of retroviral genomes in which the *gag*, *pol* and *env* genes have been removed and replaced with the gene of interest. These vectors are produced by packaging cell lines, genetically modified to express the missing structural genes. Transcription of packaging cell DNA generates mRNA encoding for the viral structural proteins. The vector RNA is then packaged with these structural proteins to form an infectious retroviral vector expressing the gene of interest. Packaging cell *gag*, *pol* and *env* structural gene sequences are modified so that, though transcribed, they cannot subsequently be packaged into virions. Retroviral vectors, therefore, lack the essential *gag*, *pol* and *env* genes and so are unable to replicate further once a target cell has been infected [22–26].

Retroviruses may infect a wide variety of cells, but they are only able to integrate and express their genome in cells actively dividing at the time of infection [27]. This is a potential advantage when considering cancer gene therapies, and retroviral vectors have been used to efficiently transduce rapidly dividing tumours which exist within tissues undergoing little or no division at all, such as brain or liver [17, 18]. In addition, because retroviral vectors are integrated within the genome, expression is possible over an extended period and the integrated genome is replicated and maintained following cell division. However, retroviral vectors have a number of potential disadvantages. Retroviral packaging cell lines produce retroviral vectors at relatively low titres, for example 10^5–10^7 colony forming units per ml of culture supernatant. In addition, the murine retroviral vectors in current clinical use are rapidly inactivated by human complement [28] and this may reduce the efficiency of gene transduction *in vivo*. However, high titre packaging cell lines and vectors resistant to human complement are now becoming available [29]. There are also a number of important safety concerns regarding retroviral vectors. Theoretically, recombination events may result in the generation of replication competent virus, a potential hazard to health [30]. Also, retroviral vectors are integrated into the host genome randomly. There is, therefore, a small but finite risk of insertional mutagenesis and oncogenic transformation. However, insertional mutagenesis has never occurred in animals or in human gene therapy subjects following the administration of replication defective retroviral vectors. Although relatively small numbers of patients have been treated with retroviral vectors, mathematical models suggest that the risk of malignant transformation secondary to insertional mutagenesis is extremely low [31].

Adenoviral vectors

Adenoviruses are double-stranded DNA viruses of approximately 36 kilobases in length. On entry into a cell, the virus is taken up by an endosome. The adenovirus disrupts the endosome leading to viral DNA release. Viral DNA transcription then results in the production of mRNA that is translated into viral structural proteins. The viral DNA also undergoes replication, enabling the formation of infective viral particles. Adenoviruses can be engineered to become replication incompetent by removal of early region genes, primarily *E1a* and *E1b*. The *E3* region is also deleted, and in a vector where the *E1a*, *E1b* and *E3* regions have been deleted, there is approximately 7.5 kb available in which to insert a gene of interest. Replication defective adenoviral vectors are produced by packaging cell lines that express the *E1a* and *E1b* regulatory genes [32].

Adenoviral vectors have a number of advantages over retroviral vectors as vehicles for gene delivery. Adenoviral vectors can be produced at high titres, for example 10^{11} colony forming units per ml. Cell division is not required for gene transfer so adenoviral vectors are able to infect a wide variety of dividing and non-dividing cells *in vivo*. In addition, adenoviral vectors do not become integrated within the host genome and so there is no risk of insertional mutagenesis. However, because adenoviral vectors cannot replicate, the expressed gene becomes increasingly dilute in a population of dividing cells. Adenoviral vectors also have a number of other important limitations. They have a tendancy to generate replication competent adenovirus by genetic recombination. Following infection with an adenoviral vector, transcription occurs not only of the delivered gene, but also of viral structural genes. The production of viral proteins may result in toxicity and the development of pathology in target organs such as the liver [33]. The expression of viral proteins also results in the induction of a powerful immune response against infected cells. As a result, only transient gene expression occurs *in vivo* and it has been impossible to successfully re-administer virus of the same serotype following an initial infection [34, 35]. Despite these limitations, it may still be possible to effectively use adenoviral vectors for

some cancer related therapies when only transient gene expression is required.

Other viral vectors

A number of other viral gene delivery systems are under development. Adeno-associated virus is a small DNA virus which appears to be able to integrate into the human genome at a specific site on chromosome 19 [32, 36]. Vaccinia virus is a large DNA virus that can be engineered to deliver genes to mammalian cells [37]. Herpes virus is a large DNA virus that infects and persists in cells of the nervous system. It is, therefore, possible that herpes virus-based vectors may provide a means of obtaining long-term gene expression in cells of the central nervous system [38].

Non-viral gene delivery systems

Non-viral methods of gene delivery include the use of cationic liposomes [39], ligand DNA conjugates [40] and adenovirus ligand DNA conjugates [41]. Currently, these non-viral gene delivery systems do not approach the efficiency of viral gene delivery systems.

SUICIDE GENES

As described above, suicide gene therapy strategies aim to create artificial differences in the sensitivity of normal and malignant cells to chemotherapeutic agents. Most suicide genes mediate sensitivity by encoding for viral or bacterial enzymes that convert a non-toxic prodrug into toxic antimetabolites capable of inhibiting DNA synthesis. The most commonly used suicide gene is the Herpes Simplex Type 1 Thymidine Kinase (*HSV1-TK*) gene. Expression of the *HSV1-TK* gene results in the phosphorylation not only of thymidine, but also a number of nucleoside analogues that are poor substrates for cellular thymidine kinase, including the guanosine analogues, acyclovir (ACV) and ganciclovir (GCV). These monophosphorylated nucleotides are subsequently converted into diphosphorylated and triphoshorylated compounds by cellular enzymes. ACV and GCV are normally used as treatments for herpes simplex virus or cytomegalovirus infection. In this context, the triphosphorylated substrate inhibits the viral DNA polymerase, preventing viral replication. Triphosphorylated forms of ACV or GCV do not inhibit eukaryotic cell polymerases and they may, therefore, be used as substrates for DNA synthesis. Incorporation of these phosphorylated substrates into the DNA of dividing cells results in DNA chain termination and cell death. The pharmacologically active forms of ACV or GCV are, therefore, only toxic to dividing cells and this is obviously an important advantage when considering cancer therapies. Moreover, the *HSV1-TK* gene is almost 1000 times more efficient at phosphorylating GCV than its cellular counterpart and, therefore, GCV is usually non-toxic to normal cells [42–47].

The cytosine deaminase (*CD*) gene is also commonly used in experimental models of suicide gene therapy. Cytosine deaminase is an enzyme found in some bacteria and fungi, but not in mammalian cells. Its normal function is to catalyse the deamination of cytosine to uracil. However, cytosine deaminase also converts the non-toxic prodrug 5-fluorocytosine to the toxic anabolite 5-fluorouracil [48–50]. 5-Fluorocytosine is commonly used as an antifungal agent, whereas 5-fluorouracil is a powerful cytotoxic agent, widely used in cancer chemotherapy. The introduction of clinical trials using suicide genes to treat cancer has been greatly facilitated by the fact that both CV and 5-fluorocytosine are in routine clinical use [51]. The pharmacology and possible side-effects of these agents are, therefore, well known.

In addition to *HSV1-TK* and cytosine deaminase, a number of other suicide gene systems have been described or are under development [52]. These include the varicella-zoster virus thymidine kinase gene [53], the *E. coli* xanthine guanine phosphoribosyl transferase (*XGPRT*) gene [54], the *E. coli* purine nucleoside phosphorylase (*DeoD*) gene [55] and the cyclophosphamide activating P450 cytochrome *2B1* gene [56].

Recently, a novel enzyme prodrug activating system has been described [57], which involves the use of the *E. coli* nitroreductase gene. CB1954, a weak monofunctional alkylating agent, is a relatively non-toxic produg which is activated by the *E. coli* nitroreductase (NTR) enzyme to form a highly toxic bifunctional alkylating agent. In contrast to the HSV1-TK/GCV suicide gene system, activated CB1954 will kill both dividing and non-dividing cells. This may be an advantage when considering the treatment of human tumours when only a relatively small proportion of cells may be actively dividing at any one time.

In addition to cancer gene therapy, suicide genes may have a number of other potential applications. For example, Caruso and associates have shown that $CD4^+$ lymphocytes, expressing the *HSV1-TK* gene under the control of HIV regulatory sequences, are protected from HIV spread by treatment of the cell culture with ACV [58]. This effect has been shown to be due both to the destruction of the initial population of HIV infected cells and to the inhibition of HIV reverse transcription by triphosphorylated forms of ACV [59]. It may also be possible to use the *HSV1-TK* gene for the treatment of graft versus host disease (GVHD) following allogeneic bone marrow transplantation. *Ex vivo*, T lymphocyte marrow depletion effectively prevents GVHD. However, this increases the risk of graft rejection and reduces the graft versus leukaemia effect that is observed. The *ex vivo* transfer of the *HSV1-TK* gene into T cells before re-infusion could allow the selective *in vivo* depletion of T cells with GCV if GVHD was found to occur [60].

EXPERIMENTAL MODELS OF SUICIDE GENE THERAPY

The HSV1-TK *gene*

The initial experiments that demonstrated the killing of tumour cells by suicide genes were performed by Moolten in 1986 [15]. Murine sarcoma cells transduced with the *HSV1-TK* gene were killed *in vitro* by GCV. *HSV1-TK* expressing cell lines were found to be greater than 1000 times more sensitive to ganciclovir than *HSV1-TK* negative cell lines. The effectiveness of this approach was also demonstrated *in vivo*. BALB/C mice bearing subcutaneous tumours expressing the *HSV1-TK* gene showed complete tumour regression when treated with intraperitoneal GCV. *HSV1-TK* negative tumours did not respond and all animals died.

Initially, it was thought that this anticancer approach could be used prophylactically in individuals at high risk of developing a particular cancer such as leukaemia. The *HSV1-TK* gene and other suicide genes would be used to

generate a mosaicism within an individual. If a tumour arose from one of these genetically modified cells, then the individual could be treated with the appropriate prodrug and the tumour could be eliminated. This approach remains somewhat impractical at present. However, this system has been successfully used for the treatment of pre-existing tumours [17–19].

Ezzedine and associates used a retroviral vector to generate a *HSV1-TK* expressing glioblastoma cell line *in vitro* [61]. The administration of intraperitoneal GCV prevented the *in vivo* growth of subcutaneous tumours expressing the *HSV1-TK* gene. The same group had previously shown that retroviral transduction of a tumour could be achieved by the direct intratumoral injection of a packaging cell line producing recombinant retrovirus. They also showed that the direct injection of the packaging cell line allowed for a transduction efficiency much larger than that resulting from injection of the viral supernatant alone [62].

Culver and colleagues [17] developed an experimental model for the *in situ* treatment of glioblastoma. Microscopic brain tumours were generated by an injection of glioblastoma cells into the frontal lobe of rats. After 7 days, the rats underwent a stereotactic intratumoral injection of packaging cells producing recombinant retrovirus expressing the *HSV1-TK* gene. A 5-day period was allowed for retroviral transduction and this was followed by 5 days of treatment with intraperitoneal GCV. Complete tumour regression was observed in 11 out of 14 rats, whereas control animals developed rapidly fatal tumours [17]. These results were somewhat surprising as the efficiency of tumour transduction was thought to be well below 100%. In order to investigate these observations, subcutaneous tumours were generated using different ratios of wild-type tumour cells and *HSV1-TK* expressing tumour cells. Treatment with ganciclovir resulted in complete tumour regression in nearly all animals inoculated with 50:50 tumour ratios and in some animals with as few as 10% *HSV1-TK* expressing cells in the inoculum. In a similar study, after injection of packaging cells producing recombinant retrovirus expressing the *HSV1-TK* gene, a 7-day period was allowed for retroviral transduction and this was followed by 14 days treatment with intraperitoneal GCV [19]. Twenty-three of the 30 rats treated showed comoplete tumour regression. In this study, control animals received a packaging cell line that produced a retroviral vector expressing the *E. coli* β-galactosidase gene. β-Galactosidase may be detected histologically following staining with X-Gal solution and an estimate of retroviral transduction *in vivo* may, therefore, be made. In control animals, it was found that between 10 and 70% of tumour cells only had undergone retroviral transduction. It, therefore, appeared that complete or near total tumour regression could be achieved even though only a proportion of the tumour had been genetically modified. In parallel experiments, Barba and associates [20] generated experimental brain tumours using different ratios of *HSV1-TK* positive and *HSV1-TK* negative tumour cells. They were also able to demonstrate the complete regression of experimental brain tumours when only 50% of the tumour cells expressed the *HSV1-TK* gene [20].

These therapies have also been applied to the treatment of experimental liver metastases. Caruso and associates were able to demonstrate the complete regression of established macroscopic liver metastases following the *in situ* transduction of the *HSV1-TK* suicide gene [18]. Liver metastases were generated by the subcapsular injection of a colon adenocarcinoma cell line. At 5 days, a further laparotomy was performed and tumours of between 2 and 3 mm diameter were visible. Tumour deposits were injected with a ψCRIP packaging cell line producing a *HSV1-TK* expressing retroviral vector. Five days were allowed for retroviral transduction and the animals were then treated with intraperitoneal GCV for a further 5 days. Overall, a 90% reduction in tumour volume was obtained, the remaining tumour being essentially fibrous. In one third of animals, tumour regression was found to be complete. Parallel studies using a packaging cell line producing an *E. coli* β-galactosidase expressing retroviral vector showed that retroviral transduction was restricted to tumour cells. There was no retroviral transduction of normal liver and there was no hepatic damage in the areas adjacent to treated tumour deposits. It was also demonstrated that only 10–20% of the tumour cells were required to take up the retroviral vector in order for 95–98% tumour regression to occur. Again, total tumour regression was achieved even though only a fraction of the tumour mass was genetically modified. This phenomenon of the death of untransduced cells is now known as the bystander effect [17].

Adenoviral vectors have also been used in experimental models of suicide gene therapy. Smythe and associates used a recombinant adenoviral vector expressing the *E. coli* β-galactosidase gene to transduce human mesothelioma cells *in vitro* [63]. *In vivo*, the introduction of recombinant adenovirus into the peritoneal cavity of severe combined immunodeficiency (SCID) mice with established intraperitoneal mesothelioma has resulted in extensive gene transfer at the tumour surface and within tumour nodules [63]. A recombinant adenoviral vector has also been used to transfer the *HSV1-TK* gene and transduce a mesothelioma cell line *in vitro*. Transduced cells were found to be approximately a 1000-fold more sensitive to GCV than uninfected cells or cells transduced with a control virus [64]. In addition, a bystander effect was observed *in vitro* with no diminution of the efficacy of GCV treatment until the ratio of infected to uninfected cells was less than 10% [64]. A strong bystander effect has also been observed *in vivo* [65]. Subcutaneous tumours were eliminated by the administration of intraperitoneal GCV even when as few as 10% of tumour cells were transduced with the *HSV1-TK* gene [65]. In a therapeutic model for human mesothelioma, the administration of recombinant adenovirus into the peritoneal space of mice with established tumour, followed by GCV therapy, resulted in the eradication of microscopic tumour in eight out of ten animals. Control animals were found to have bulky macroscopic intraperitoneal disease [65].

Chen and colleagues [66] also used a recombinant adenoviral vector expressing the *HSV1-TK* gene to transduce experimental gliomas both *in vitro* and *in vivo*. *In vivo*, tumours were generated by the stereotactic intracerebral injection of C6 glioma cells into nude mice. After 8 days of tumour growth, recombinant adenovirus was injected into the tumours and after 12 h, animals were treated with intraperitoneal GCV for 6 days. Tumour volume in treated and control animals was compared after a total of 20 days. The mean cross-sectional area of the tumours in the treated group was 23-fold smaller than in control animals. This was equivalent to a 500-fold reduction in tumour volume.

In murine models, recombinant adenoviral vectors expressing the *HSV1-TK* gene have also been used for the treatment of squamous cell carcinoma of the head and neck [67], malignant melanoma [68] and hepatocellular carcinoma [69]. It, therefore, appears that *HSV1-TK*/GCV mediated tumour cell death and the generation of the bystander effect are independent of the method of gene delivery, having been demonstrated using both retroviral and adenoviral gene delivery systems.

Other suicide genes

The gene for cytosine deaminase was first cloned in 1992 [48]. It was then possible to genetically modify tumour cells to express cytosine deaminase, and these were shown to be sensitive to 5-fluorocytosine *in vitro* [49, 50]. Subcutaneous tumours, expressing the cytosine deaminase gene, were also shown to regress following the intraperitoneal administration of 5-fluorocytosine into nude mice [50, 70]. In the *in vitro* studies, a bystander effect was not initially observed with cytosine deaminase, but a bystander killing has clearly been shown in subsequent studies [71].

Bridgewater and associates used a retroviral vector to transduce NIH3T3 cells with the *E. coli* nitroreductase gene [57]. NIH3T3 cells expressing the *NTR* gene were sensitised to CB1954. It was also possible to demonstrate the presence of a bystander effect *in vitro*. A 90% reduction in [^{3}H]thymidine incorporation was observed even though only 50% of cells expressed the *NTR* gene. Human ovarian carcinoma, melanoma and mesothelioma cell lines were also shown to be sensitive to CB1954 following transduction of the *NTR* gene.

Few studies have compared the efficiency of the bystander killing that occurs with different suicide gene systems. Trinh and associates compared the *HSV1-TK* and the cytosine deaminase suicide gene systems. They treated subcutaneous tumours composed of mixed cell populations in which only a proportion of the cells expressed the therapeutic gene. A greater bystander effect was found with the *CD*/5-FCyt system when compared to the *HSV1-TK*/GCV system [72]. Phosphorylated GCV is unable to cross cell membranes, whereas 5-fluorouracil crosses cell membranes by passive diffusion [73]. It has, therefore, been proposed that 5-fluorouracil causes a more powerful bystander effect because of its ability to diffuse to nearby tumour cells and directly kill them. However, since this study was performed in nude mice, using a cell line that did not possess gap junctions, this may not be a fair comparison. Given what is known of the mechanism of the bystander effect both *in vitro* and *in vivo* (see below), further studies will be required in order to compare the relative efficiency of the bystander effect in these two suicide gene systems.

Bridgewater and associates isolated a cell line that expresses both the nitroreductase and *HSV1-TK* genes [57]. When the sensitivity of this cell line to the two prodrugs was compared, GCV was found to be approximately 4-fold more effective at cell killing than CB1954. If the two prodrugs were added in combination, an additive effect on cell killing was observed. Furthermore, it was also found that the combined use of CB1954 and GCV resulted in an improved bystander killing effect. Single drug treatment required 30–50% of cells to express the *NTR* and *HSV1-TK* genes for an overall 90% cell killing to be observed. However, when a combination of CB1954 and GCV was used, 90% of cells were killed when only 10% of the cell population expressed the *NTR* and *HSV1-TK* genes [57].

Targeting of gene expression

One of the potential problems when considering the use of suicide genes for cancer therapy, is how to restrict suicide gene expression to tumour and therefore, prevent toxicity in normal tissues. This may be achieved in part on the basis of cellular proliferation, as retroviral vectors only target dividing cells. However, adenoviral vectors target both dividing and non-dividing cells and the use of retroviral vectors may still result in toxicity if surrounding tissues are undergoing cell division. One possible solution is the use of tissue- or tumour-specific promoters that will restrict gene expression to the target cell population. Vile and Hart have used the 5′ flanking region of the murine tyrosine kinase gene to direct expression of both a reporter gene [74] and the *HSV1-TK* gene [75]. Gene expression was found to be restricted to human and murine melanoma cells and melanocytes whereas expression in a number of other cell types did not occur. The carcino-embryonic antigen (CEA) and α-fetoprotein promoter regions have both been used to drive expression of the *HSV1-TK* gene [76, 69]. Following transfection, only lung adenocarcinoma cell lines and hepatocellular carcinoma cell lines expressing the relevant tumour marker were found to be sensitive to GCV. Tumour specific gene expression has also been achieved in human breast carcinoma cell lines using the *C-ERB-2* [77] and the *DF3* gene [78] promoter regions.

THE BYSTANDER EFFECT

A number of theories have been proposed to explain how the bystander killing of neighbouring cells occurs. The phenomenon of the death of untransduced cells was initially described by Moolten and associates [15]. When 9:1 mixtures of *HSV1-TK* positive and negative cells were plated sparsely and allowed to grow to confluence, ganciclovir treatment destroyed *HSV1-TK* positive cells leaving *HSV1-TK* negative patches to survive. However, then 9:1 mixtures were plated at high density so that each *TK* negative cell was surrounded by *HSV1-TK* positive cells then only rare *TK* negative cells survived GCV treatment. Cell–cell contact appeared to be required for the bystander effect to be observed *in vitro* and it was suggested that this may be mediated by the transfer of phosphorylated GCV between cells by metabolic co-operation.

Metabolic co-operation was first described in experiments showing that HPRT deficient cells could not incorporate [^{3}H]hypoxanthine when cultured in isolation, but did so when in contact with IPP positive cells [79]. An association was later established between metabolic co-operation, ionic coupling and the occurrence of gap junctions by electron microscopy [80]. Cells with gap junctions were ionically coupled and participated in metabolic co-operation while cells that lacked gap junctions did not [80, 81].

Gap junctions couple cells via channels which permit the passage of small molecules with molecular weights below a thousand daltons [82]. Second messengers, such as cAMP and calcium ions pass freely through these junctions as do many metabolites. GCV is readily permeable to cells but its phosphorylated metabolite cannot passively diffuse across cell membranes. The molecular weight of phoshorylated GCV is approximately 400, well within the limit of around

1000 daltons permissible for the passage of molecules through gap junctions [82]. It is, therefore, possible that phosphorylated GCV may be transferred to neighbouring cells via gap junctions and exert a cytotoxic effect on cells not expressing the *TK* gene.

Bi and coworkers tested this hypothesis [83]. In co-culture experiments, *TK* negative human fibrosarcoma cells were found to be killed by GCV, but only when in contact with *TK* positive cells. In addition, *TK* negative cells were shown to accumulate labelled GCV, but only when in contact with a *TK* positive cell. The much larger molecule β-galactosidase was not transferred from β-Gal positive cells to β-Gal negative cells, irrespective of whether or not there was cell–cell contact [83].

Freeman and associates also performed experiments in which *HSV1-TK* positive cells and *HSV1-TK* negative cells were mixed *in vitro* at various ratios [16]. They found that if as few as 10% of the mixed cell population was *HSV1-TK* positive, then the majority of the population was eradicated following exposure to GCV. The tumoricidal effect of *HSV1-TK* positive cells on *HSV1-TK* negative cells was completely abolished when the two cell populations were separated by a filter membrane. It was, therefore, thought that this bystander killing could not be due to the release of a toxic soluble factor by *HSV1-TK* positive cells. In this study, *HSV1-TK* positive cells, dying following exposure to GCV, were analysed by microscopy. These cells exhibited cell shrinkage, cell detachment, vesicle formation and chromatin condensation, all characteristic features of apoptosis. Ultrastructural features of apoptosis were also confirmed by transmission electron microscopy. A further potential mechanism that would account for the bystander effect is the release of apoptotic vesicles by dying tumour cells. Apoptotic vesicles could transfer the toxic metabolite of GCV or the *HSV1-TK* enzyme itself and tumour cells are known to phagocytose nearby apoptotic vesicles. Clearly close cell–cell contact would be required for this effect to be seen. Using a fluorescent tracking dye, fluorescence microscopy and flow cytometry, Freeman and associates were able to show that *HSV1-TK* negative cells were able to take up a label associated with the cytoplasmic vesicles generated by dying *HSV1-TK* positive cells [16]. These findings were also confirmed by transmission electron microscopy, thereby establishing a further potential mechanism for the generation of the bystander effect *in vitro*.

AN IMMUNOLOGICAL COMPONENT TO THE BYSTANDER EFFECT *IN VIVO*

Metabolic co-operation and the release of apoptotic vesicles are both implicated in the mediation of the bystander effect *in vitro*. The relative contribution of these two effects is probably dependent on whether or not cells possess gap junctions. However, there is increasing evidence to suggest that the immune system has an important role to play *in vivo*.

Columbo and colleagues [84] performed co-culture experiments and showed that *TK* positive and *TK* negative glioma cells were both killed following the administration of GCV. This was found to be associated with apoptotic cell death. Connexins, which are major components of gap junctions [85], were also detected in these cell lines, suggesting that metabolic co-operation could also contribute to the bystander effect that they had observed *in vitro*. However, when 1:1 ratios of *HSV1-TK* positive and *HSV1-TK* negative tumour cells were inoculated subcutaneously in nude mice, it was found that the effects observed *in vitro* were insufficient to prevent tumour growth occurring in immunodeficient animals.

A number of authors have described the presence of an intense inflammatory infiltrate in the regressing tumours of immunocompetent animals treated using either the *HSV1-TK* or cytosine deaminase suicide genes [18, 20, 86]. Caruso and associates [18] demonstrated an active local immune response with a massive infiltration of regressing tumours by macrophages and lymphocytes. Other authors have commented on how *HSV1-TK* transduced tumours were less responsive to GCV in athymic mice when compared to immunocompetent animals [21, 87, 88]. These findings also suggest that the immune system may have a role to play in tumour regression *in vivo*.

Tapscott and colleagues [89] found that foreign gene products can inhibit tumour formation even in the absence of selection. Animals receiving intracerebral injections of 9L glioma cells expressing the *HSV1-TK* gene showed prolonged survival with or without the subsequent administration of ganciclovir. Furthermore, subcutaneous tumours generated using the *HSV1-TK* positive cell line were found to be smaller than tumours generated from untransduced cells. Similar findings were also observed when a 9L glioma cell line expressing the neomycin resistance gene was used to generate subcutaneous tumours. The 9L glioma cell line is known to be immunogenic [90]. However, these observations suggest that the expression of a foreign protein further enhanced the immunogenicity of this cell line.

In our laboratory, we have demonstrated an immunological component to the bystander effect *in vivo* [21]. Subcutaneous tumours were generated by the co-injection of a murine colon carcinoma cell line and a packaging cell line producing a retroviral vector expressing the *HSV1-TK* gene. The administration of GCV resulted in an overall 90% reduction in tumour volume when compared to controls. Parallel experiments using a β-galactosidase expressing retroviral vector showed that only 10–20% of the tumour cells had undergone retroviral transduction, indicating that a strong bystander effect was operative. However, nude mice were greatly impaired in their ability to effect tumour regression, suggesting a strong cell mediated immune component to the bystander effect *in vivo*. In addition, immunofluorescent studies showed the presence of a pedominately CD8 positive T cell infiltrate in the regressing tumours in immunocompetent animals.

In an earlier report, Vile and associates [87] used an *HSV1-TK* expressing retroviral vector, driven by the tissue-specific tyrosine kinase promoter [74, 75], to treat recently established lung metastases of B16 melanoma. Animals received multiple intravenous administrations of high titre retroviral supernatant followed by the administration of GCV. The metastatic burden was consistently decreased by up to 90% in the lungs of immunocompetent mice when compared to control animals. However, no statistically significant reduction was observed in nude mice [87].

Freeman and colleagues [88] also showed that an intact immune system is required for an *in vivo* bystander effect to be seen. Nude mice and sublethally irradiated mice failed to show the regression of subcutaneous tumours when the tumour population was composed of 50% *HSV1-TK* posi-

tive cells. In contrast, immunocompetent mice showed tumour rejection upon receiving the same number and proportion of *HSV1-TK* positive cells. In a further *in vivo* model, an established intraperitoneal *HSV1-TK* negative tumour was treated by the administration of *HSV1-TK* positive cells, followed by treatment with ganciclovir. Tumour necrosis was found to occur much more quickly than the toxic effect of *HSV1-TK* positive cells on nearby unmodified cells observed *in vitro*. This suggested an alternative method for the generation of the bystander effect *in vivo*. Tumours were analysed for the presence of a number of cytokines by reverse transcriptase PCR. The mRNA to TNF-α, IL-1α and IL-6 was detected 24 h following the onset of treatment. Subsequent analysis demonstrated a cytokine cascade effect, with INF-γ being detected at 48 h and GM-CSF being detected at 96 h. The presence of TNF-α protein was confirmed by immunohistochemical staining. Further immunohistochemical staining also showed the presence of a macrophage and T cell infiltrate in the regressing tumours of immunocompetent mice treated with *HSV1-TK* positive cells and GCV [88]. It appears that the death of *HSV1-TK* positive cells, following the administration of GCV, is associated with the generation of an immunostimulatory intratumoral cytokine cascade and the recruitment of inflammatory cells. These would appear to be optimal conditions for the generation of an antitumour immune response.

SYSTEMIC ANTITUMOUR IMMUNITY FOLLOWING SUICIDE GENE THERAPY

In support of the above, a number of studies have now clearly shown the development of a systemic, tumour-specific, antitumour immunity following the treatment of localised tumours by suicide gene therapy.

Vile and colleagues [87] showed that, *in vivo*, the killing of *HSV1-TK* expressing melanoma cells by treatment with GCV resulted in protection against rechallenge with wild-type tumour. This antitumour immunity appeared to be tumour-specific as treated animals remained just as susceptible to challenge with antigenically unrelated tumour cell lines. However, this protective immunity was only partial in that tumour growth did eventually occur, but only after a significant delay compared to other treatment groups.

Other authors demonstrated the development of long-term systemic antitumour immunity following the *in vivo* killing of tumour cells using either the *HSV1-TK* [91] or the cytosine deaminase suicide gene systems [70].

Barba and colleagues [91] treated experimental brain tumours by the intratumoral injection of a packaging cell line producing an *HSV1-TK* expressing retroviral vector, followed by the administration of GCV. In long-term studies, 22% of animals survived 90 days. Histological examination of the brains of successfully treated animals demonstrated a small number of residual tumour cells which were associated with an inflammatory infiltrate consisting of macrophages and T cells. The presence of residual tumour cells in the brains of treated animals suggested that antitumour immunity may have been an important factor in the sustained tumour regression that was observed. In addition, surviving rats were able to completely reject repeat tumour injections into the contralateral brain or flank at doses that consistently resulted in tumours in naive animals [91].

Mullen and associates showed that cytosine deaminase expressing subcutaneous fibrosarcomas or adenocarcinomas were eradicated *in vivo* following the systemic administration of 5-fluorocytosine. Treated animals subsequently resisted rechallenge with tumorigenic doses of wild-type tumour. This antitumour immunity was also found to be tumour-specific. The eradication of a cytosine deaminase expressing subcutaneous adenocarcinoma conferred no protection to rechallenge with wild-type fibrosarcoma [70].

Similar results were obtained by Consalvo and colleagues [88]. They used a cell line derived from a spontaneously occurring mammary tumour, rather than a carcinogen-induced tumour cell line. The 5-fluorocytosine induced regression of cytosine deaminase-expressing subcutaneous tumours resulted in protective immunity. Treated mice were able to resist both the subcutaneous injection and the administration of a normally lethal intravenous dose of wild-type tumour [88].

This group went on to perform *in vivo* antibody depletions in order to more clearly define the cellular components of the immune system that contribute both to tumour regression and to the antitumour immunity subsequently demonstrated. The initial 5-fluorocytosine-induced regression of cytosine deaminase-expressing subutaneous tumours was found to be mediated by CD8 positive lymphocytes, with depletion of this T cell subset resulting in unrestrained tumour growth. The depletion of granulocytes also abolished tumour regression, although many tumours subsequently regressed when the administration of anti-granulocyte monoclonal antibody was interrupted. Depletion of CD4 positive T cells did not impair tumour regression and all mice eventually rejected tumours. However, the depletion of CD4 positive T cells completely prevented the induction of systemic antitumour immunity. Antibody depletions were also performed during the effector phase of tumour rejection. Separate removal of CD4 positive or CD8 positive T cells reduced the ability to reject a repeat tumour challenge to approximately 70–80%, but no resistance remained when both CD4 positive and CD8 positive T cells were removed. Morphologically, when rechallenged, rejected tumours were predominantly infiltrated by neurophils with relatively few lymphocytes, suggesting an important role for neutrophils when considering the effector phase of antitumour immunity [88].

EVIDENCE FOR THE EXISTENCE OF A 'DISTANT BYSTANDER EFFECT'

It is now clear that the immune system has an important role to play in the regression of established tumours treated by suicide gene therapy, and that this may result in the development of a systemic, tumour-specific antitumour immunity. This raises the question as to whether the eradication of localised tumour deposits will result in the simultaneous immune mediated regression of anatomically distant metastases. There is now some evidence for the existence of such a 'distant bystander effect'.

Initially, the bystander effect was thought to be a localised phenomenon. In murine studies, the treatment of a cytosine deaminase or a thymidine kinase expressing subcutaneous tumour on one flank was found to have no effect on the size or progression of a subcutaneous wild-type tumour on the opposite flank [16, 50, 86, 70]. Thus, the regression of cytosine deaminase or thymidine kinase-expres-

sing tumours appeared to be mediated by a localised intratumoral immune response.

In a therapeutic model for ovarian carcinoma, it was shown that increased survival could be achieved following the intraperitoneal administration of an irradiated xenogeneic *HSV1-TK* expressing tumour line and treatment with GCV [92]. It was thought that this bystander effect was mediated by cell–cell contact within the peritoneal cavity. These observations led to the development of a clinical protocol for the treatment of patients with intraperitoneal ovarian carcinomatosis [93]. However, as described above, it is now clear that these effects are mediated in part by the generation of a cytokine cascade and an antitumour immune response [88].

Other authors have reported the regression of anatomically distant metastases following the eradication of a localised CD or *TK* expressing tumour deposit. Consalvo and colleagues showed that 20% of mice bearing 4-day old lung metastases were cured following the 5-fluorocytosine-induced regression of a cytosine deaminase-expressing subcutaneous tumour [86]. This was thought to be mediated by the generation of a systemic antitumour immune response.

Misawa and associates [94] demonstrated the regression of *HSV1-TK* negative liver metastases following the eradication of a *HSV1-TK* positive intraperitoneal tumour burden. Intraperitoneal tumours were generated by the injection of a *HSV1-TK* positive or *HSV1-TK* negative colon adenocarcinoma cell line. After 10 days, all animals received an intrahepatic injection of *HSV1-TK* negative cells as a second tumour challenge. Following the administration of GCV, animals bearing *HSV1-TK* positive intraperitoneal tumours were found to show an overall 90% reduction in the mean tumour volume of *HSV1-TK* negative hepatic tumours when compared to controls.

Professor Klatzmann's group treated animals with two colorectal liver metastases. One tumour was *HSV1-TK* positive and the other was *HSV1-TK* negative. Following the administration of GCV, both the *HSV1-TK* positive and the *HSV1-TK* negative tumours were found to regress. Both tumours were infiltrated by inflammatory cells and histologically there was no detectable difference between the residual *HSV1-TK* positive and *HSV1-TK* negative tumours (Professor Klatzmann, Hôpital de la Pitié Salpétrière, Paris, France).

These observations suggest that, under certain experimental conditions, there is a systemic immune component to the bystander effect, capable of mediating the regression of distant metastases. Interestingly, this effect appears to be influenced by anatomical location. A second wild-type subcutaneous tumour is not affected by prodrug administration even though animals are able to subsequently reject a repeat tumour challenge. However, as described above, untransduced liver and lung metastases have been shown to undergo regression at the time of prodrug administration. These differences may be due to the way in which antigens are presented to the immune system in different organs or due to the increased number of cells of the reticuloendothelial system in organs such as the liver or lung. Clearly, if this effect could be enhanced, this could have major implications for the prognosis of patients with occult microscopic liver or lung metastases who would subsequently relapse following conventional treatment.

COMBINATION CYTOKINE AND SUICIDE GENE THERAPY

Tumour cells may be engineered *in vitro* to secrete a variety of cytokine genes. Cytokine gene therapy involves the inoculation of these genetically modified cells into immunocompetent syngeneic animals. The expression of a wide variety of genes, including IL-2 [11, 95–99], IL-4 [98, 101], INF-γ [98, 99, 102, 103], IL-7 [104], TNF-α [105], GM-CSF [106] and G-CSF [107, 108], has resulted in decreased tumorigenicity and enhanced immunogenicity. Cytokine-producing tumour cells are rejected. Initially, this appears to be mediated by a local rather than a systemic immune response, as injection of a cytokine-producing tumour on one flank does not inhibit the growth of wild-type tumour on the opposite flank [11, 100, 103, 105]. However, several groups have established that the inoculation of a cytokine-secreting tumour cell line results in the development of an antigen-specific systemic immunity against the wild-type tumour cell line [11, 101, 103]. *In vitro* and *in vivo*, the antibody depletion of T cell subsets has shown that cytotoxic T lymphocytes have a central role to play in tumour rejection [11, 96, 98, 101, 105, 106, 108]. In many respects, there appear to be parallels between the immunity generated by cytokine gene therapy and the immunity that is observed following the eradication of *CD* or *HSV1-TK* expressing tumour deposits. It is possible that the therapeutic benefits of suicide gene therapy could be enhanced by the combined use of suicide genes and vectors that encode cytokine genes. This approach has been investigated in a number of model systems.

In an experimental model for the treatment of glioblastoma, Ram and colleagues achieved tumour eradication in 50% of rats treated by the intratumoral injection of *HSV1-TK* vector producer cells followed by the administration of intraperitoneal GCV. However, when the *IL-2* gene was combined with the *HSV1-TK* gene, they found that there was no enhancement of tumour eradication [109]. In parallel experiments, it was found that the transduction of glioma cells with the *IL-2* gene suppressed the growth of subcutaneous tumours, but failed to influence the growth of brain tumours. They concluded that there would appear to be only a limited role for immune enhancement by transduction with *IL-2* either alone or in combination with *HSV1-TK* when considering the treatment of experimental brain tumours.

Chen and colleagues [110] reported enhanced tumour regression when using combined cytokine and suicide gene therapy in a murine model for the treatment of colorectal liver metastases. Liver metastases were generated by the intrahepatic injection of a colon carcinoma cell line. After 7 days, various recombinant adenoviral vectors were directly injected into hepatic metastases. The mice were then treated intraperitoneally with GCV for 6 days. Tumours continued to grow in all animals treated with a control β-galactosidase expressing vector or a murine *IL-2* vector. Animals treated with a *HSV1-TK*-expressing vector showed tumour regression and necrosis. However, the combined use of the *HSV1-TK* and *IL-2* vectors resulted in an enhancement of tumour regression and massive tumour necrosis. In this study, treatment with the *HSV1-TK* vector failed to generate any degree of systemic antitumour immunity. However, animals treated with the *HSV1-TK* and *IL-2* vectors were found to develop immunity to subsequent tumour challenge

at distant sites. Again, this immunity was found to be tumour-specific. Treated animals rejected the colorectal carcinoma cell line, but remained susceptible to rechallenge with tumorigenic doses of a syngeneic breast tumour cell line. Using T cell cytotoxicity assays and *in vitro* antibody depletions, it was shown that this immunity was associated with a tumour-specific population of CD8 positive cytotoxic lymphocytes. Despite the fact that it was possible to demonstrate antitumour immunity following the combined treatment with *HSV1-TK* and *IL-2* vectors, no survival benefit was observed in this study. However, in a further study, animals were treated with a combination of vectors encoding for *HSV1-TK*, *IL-2* and *GM-CSF*. In this study, 25% of animals developed long-term antitumour immunity and survived without tumour recurrence [111]. The same group used a similar approach for the treatment of squamous cell carcinoma of the head and neck. It was again shown that the combined use of the *HSV1-TK* and *IL-2* vectors resulted in enhanced tumour regression when compared to animals treated with the *HSV1-TK* vector alone. This study was also able to demonstrate a statistically significant survival benefit following the combined use of the *HSV1-TK* and *IL-2* vectors alone [112].

A PROPOSED MECHANISM FOR THE GENERATION OF THE BYSTANDER EFFECT *IN VIVO*

Experimental models of cytokine and suicide gene therapy have shown that tumour cell populations may be recognised and destroyed by the immune system [11, 21, 86, 87, 103]. In recent years, the existence of tumour associated antigens (TAAs), which may serve as targets for an immune response, has been demonstrated for several spontaneously occurring tumours [113, 114]. Tumours are generally able to grow progressively and so a variety of hypotheses have been proposed to explain why tumours grow if they are, indeed, antigenic. These include the emergence of antigen loss variants that do not display TAAs [115], the secretion of soluble inhibitory factors such as TGF-β and IL-10 by tumour cells [116], suppressor cell effects [117], the downregulation of MHC complex expression preventing the presentation of peptide antigens to potentially reactive T cells [118] or the induction of peripheral tolerance or energy to tumour cells that fail to deliver costimulatory signals [13, 119–121].

Peptide antigens are presented to CD4 positive T helper cells in association with class II MHC molecules. However, costimulatory molecules such as B7 [122] are required to provide a further signal. The costimulatory signal from B7 results in the stabilisation of mRNA for a number of T cell derived cytokines [123]. MHC restricted antigen presentation to CD4 positive T helper cells without the presence of a costimulatory signal is found to result in the inactivation of T cells. The majority of solid tumours lack class II and B7. They are, therefore, unable to present antigens to T helper cells or to provide the costimulatory signals necesary for T cell activation. Tumour-specific T helper cells become inactivated and are then unable to generate cytokines such as *IL-2*, necessary for the clonal expansion, maturation and activation of CD8 positive T cytotoxic cells. The expression of cytokine genes in tumour cells is effectively thought to bypass T helper function in the generation of an antitumour immune response, allowing he clonal expansion and activation of tumour specific CTLs [11].

The tumour regression associated with suicide gene therapy has been shown to be mediated by the immune system and to result in the generation of systemic, tumour-specific antitumour immunity. In addition, tumour regression may be associated with the regression of anatomically distant metastases. The regression of localised tumours treated by suicide gene therapy appears to be mediated by CD8 positive CTLs. However, the induction and effector phases of systemic immunity appear more complex and involve CD4 positive T helper cells, antigen presenting cells and neutrophils in addition CTLs. Currently, little is known of the immunological mechanisms that may contribute to the regression of distant metastases.

Suicide gene therapy results in cell death by apoptosis and necrotic degeneration and this appears to influence the way in which tumour antigens are presented to the immune system. Cells dying by apoptosis are known to produce IL-1 [124]. Ramesh and associates also showed that the exposure of *HSV1-TK* positive cells to GCV results in the generation of an intratumoral cytokine cascade [88]. Initially TNF-α, IL-1 and IL-6 were detected. TNF-α is known to activate a variety of cells such as macrophages, natural killer cells and cytotoxic lymphocytes, all of which may have antitumour activity. IL-1 potentiates the clonal activation of T cell populations and promotes the adhesion of T cells, monocytes and neutrophils to endothelial cells. As described above, the local production of cytokines may bypass T helper function and result in the activation of tumour-specific CTLs. Local cytokine production may also lead to tumour infiltration by other effector T cells and macrophages. Activated T cells and macrophages in turn produce cytokines leading to the generation of a cytokine cascade and the further activation of an antitumour immune response. Tumour cell death will also lead to the sudden and massive release of tumour antigens which may then be effectively presented to the immune system. In addition, thymidine kinase and cytosine deaminase are both immunogenic. It is, therefore, possible that these proteins act as superantigens, leading to the polyclonal activation of effector T cells, some of which may cross react with tumour.

CONCLUSION

Further work will be required to define more clearly the cell populations and cellular interactions that contribute to the immunological component of the bystander effect *in vivo*. This will allow the identification of the most appropriate cytokines for use in combination gene therapy protocols. It may then be possible to enhance the bystander effect and thereby increase the therapeutic efficacy of future suicide gene therapy protocols. Human gene therapy remains in its infancy. However, only further phase I and phase II clinical trials will determine if these exciting experimental observations can be translated into novel cancer therapies.

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