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EDITORIAL

Gene therapy of liver cancer

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Abstract

The application of gene transfer technologies to the treatment of cancer has led to the development of new experimental approaches like gene directed enzyme/pro-drug therapy (GDEPT), inhibition of oncogenes and restoration of tumor-suppressor genes. In addition, gene therapy has a big impact on other fields like cancer immunotherapy, anti-angiogenic therapy and virotherapy. These strategies are being evaluated for the treatment of primary and metastatic liver cancer and some of them have reached clinical phases. We present a review on the basis and the actual status of gene therapy approaches applied to liver cancer.

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Key words: Gene therapy; Cancer; Liver; Hepatocellular carcinoma; Vector; Therapeutic gene

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INTRODUCTION

Knowledge of molecular mechanisms governing malignant transformation brings new opportunities for therapeutic intervention against cancer using novel approaches. One of them is gene therapy. This new discipline is based on the transfer of genetic material to an organism with the aim of correcting a disease. The genes can be delivered directly into the subject, using a variety of vehicles named vectors (*in vivo* gene therapy), or delivered into isolated cells

in vitro that are subsequently introduced into the organism (*ex vivo* gene therapy).

Cancer is the most frequent application of experimental gene therapy approaches^[1] for several reasons.

First, the genetic alterations that give rise or contribute to the malignant transformation of cells are being unravelled with increasing detail in the last two decades, and this provides multiple candidate targets for gene therapy intervention^[2]. Nevertheless, the genetic and epigenetic alterations that lead to an established tumor are complex and require special approaches that often differ from gene therapy applied for hereditary monogenic diseases. In many cases, the transfer of genes into malignant cells is not performed with the intention of correcting a genetic deficiency related to cancer. To be efficient, this would require that the selected gene plays a dominant role in the malignant phenotype. In addition, a technique would be needed that achieves successful modification of virtually every cell in the tumor, something that is far from being realistic in the near future. Therefore, different strategies have been developed to introduce genes that cause the destruction of the tumor by indirect mechanisms, as will be discussed below.

Second, some tools initially developed as gene therapy vectors, such as certain viruses, are being exploited as oncolytic agents by themselves. This means that the development of gene therapy approaches against cancer is activating other fields such as virotherapy and cellular therapies.

Finally, the lack of safe and efficient therapeutic options against many types of cancer is fostering the development of new gene therapy applications for these diseases. Liver cancer is a good example of this situation. Hepatocellular carcinoma (HCC) accounts for 80% of primary liver tumors in adults, has an increasing incidence^[3] and a poor 5-year survival rate of about 7% despite treatment^[4]. In addition, the liver is the most frequent site of metastasis, especially from gastrointestinal cancer. Potentially curative therapies such as liver transplantation and surgical resection can only be applied to a minority of subjects because of advanced disease at the time of diagnosis and the lack of suitable organ donors. Other regional treatments may be beneficial for unresectable HCC, but recurrence is frequent and the long term survival rate remains poor. These treatments include transarterial embolization (TACE), percutaneous Ethanol injection, radiofrequency thermal ablation, microwave coagulation therapy, laser-induced thermotherapy and radiotherapy. New protocols based on combinations of regional treatments (with or without previous surgery) are being investigated for the management of HCC, and they are showing a clear advantage when compared to single treatments^[5]. In this context,

gene therapy could be considered as a potential adjuvant to other therapies. The clinical trials performed so far have shown that the side effects are acceptable in most of the cases, and that the mechanism of action is different from standard treatments^[6]. Therefore, choosing the right combination among gene therapy approaches and conventional treatments may achieve a synergistic effect. Furthermore, the refinement of interventional therapies for HCC such as TACE and PEI provides new possibilities for the delivery of gene therapy vectors into hepatic tumors, increasing the effective dose and minimizing potential side effects derived from non-target cell transduction.

In this review we present an insight into gene therapy strategies against liver cancer and discuss the latest developments in the field.

RESTORATION OF TUMOR SUPPRESSOR GENES

This strategy is the most intuitive application of gene therapy for the treatment of HCC and other cancers. It is clear that the loss of function of certain genes (caused by deletions, mutations, promoter inactivation or other epigenetic changes) is associated with malignant transformation of cells^[7]. These tumor suppressor genes control cell proliferation and apoptosis in order to maintain an equilibrated turnover of cells in each tissue. Under experimental conditions (mostly *in vitro*), it has been demonstrated that the restoration of tumor suppressor genes can revert the malignant phenotype of cells^[8]. However, the therapeutic application of this observation faces enormous difficulties. Cancer cells often suffer some degree of genetic instability. When they lose their capacity to sense and repair damaged genes, mutations accumulate and cells with higher proliferation rate and lower sensitivity to apoptotic stimuli are selected sequentially. Under these circumstances, they may become insensitive to the restoration of a particular tumor suppressor gene. On the other hand, this approach requires the introduction of the gene and the expression of the antitumoral protein in virtually all cancer cells, or at least in those responsible for tumor maintenance. This is technically impossible with current gene therapy vectors, especially for solid tumors like HCC.

Despite all these considerations, the transfer of p53 tumor suppressor gene has shown effect in several animal models of cancer, including HCC^[9,10]. This proof of concept has stimulated the use of p53 as a therapeutic gene. Mutations in p53 or alterations in its pathway have been described in more than 50% of human cancers^[11,12]. When cells lack functional p53, they are unable to stop the cell cycle or trigger apoptosis in response to DNA damage. They accumulate mutations that lead to malignant initiation, progression and resistance to treatments. Thus, the restoration of p53 may render tumor cells sensitive to apoptotic stimuli, even if they have accumulated other mutations. This may explain the therapeutic effect observed in pre-clinical models, and suggests a potential role of p53 as an adjuvant to conventional therapies that induce apoptosis in cancer cells.

In contrast, several clinical trials based on delivery of the wild type p53 gene using different vectors have observed variable, and often less positive results in different types of cancer such as lung, head and neck, bladder, ovarian and breast cancer^[13]. However, a first-generation adenoviral vector expressing the p53 cDNA under the control of the CMV promoter became the world's first commercially licensed gene therapy product (Gendicine) for the treatment of head and neck squamous cell carcinoma in China^[14]. In a clinical trial performed on 30 HCC patients, Partial Response (PR) was reported in 2 cases, Stabilized disease (SD) in 24 patients and Progressive Disease (PD) in 4 of them. The virus was administered at doses of $1-2 \times 10^{12}$ VP/wk for 4 wk. In another HCC clinical trial, Gendicine was used in combination with TACE (5FU, HCPT and ADM). The viral doses were similar ($1-4 \times 10^{12}$ VP/wk for 4 wk), starting 2-5 d after TACE. The authors reported 67.6% PR in the combination group versus 51.2% in the TACE-only group (reviewed by Peng^[14]). The clinical significance of these results is controversial at this time, but the availability of a gene-based therapy in the market with potential effect on HCC will probably extend its use in combination with other therapies and allow the identification of synergistic effects. Optimization of the vectors and the therapeutic regimes may be needed to increase gene transfer. For instance, weekly injections of a first-generation adenoviral vector are most likely eliciting a strong immune response that blocks the infectivity of repeated doses after 2 wk. A better understanding of genetic alterations in each particular case of cancer will help to predict the response to the restoration therapy and aid in the selection of candidate patients.

In addition, new strategies are being developed to address the limitations of this approach. One of them relies on the fusion of p53 with VP22, a tegument protein from Herpes Simplex Virus-1 (HSV-1)^[15]. VP22 is exported from the cytoplasm of the expressing cell and gets incorporated by neighbouring cells by poorly defined mechanisms. The fusion of VP22 with other polypeptides enables the intercellular spread of the chimeric protein. It has been demonstrated that an adenoviral vector expressing the p53-VP22 fusion construct achieves higher transduction efficiency and therapeutic effect on a rat model of HCC when compared with wild type p53^[16]. In addition, the combination of p53 with other tumor suppressor genes like p16 can cooperate to induce apoptosis in cancer cells^[17].

The suppressor of cytokine signalling 1 (SOCS-1) gene has been recently identified as a potential tumor suppressor for HCC. Its promoter is frequently inhibited by methylation in HCC, causing activation of the JAK/STAT pathway^[18]. At least *in vitro*, the restoration of SOCS-1 function induces apoptosis in cancer cells.

INHIBITION OF ONCOGENES

The rationale of this approach is in line with the previous one. In this case the correction of the imbalance between positive and negative proliferation signals is attempted by inhibiting the function of genes implicated in the maintenance of cell growth.

nance of unrestricted cell proliferation and acquisition of metastatic phenotype. Many of the drawbacks mentioned above can be applied here, like the need for a highly efficient gene transfer method and a dominant role of the target gene in malignant transformation. The number of candidate oncogenes is continuously expanding as the knowledge of cancer at the genomic and proteomic levels advances^[2].

Hopefully, the inhibition of oncogene expression will not only decrease cell proliferation, but also restore sensitivity of cells to apoptotic stimuli. For instance, it is known that the inhibition of the Ras oncogene, apart from blocking a cascade of mitotic signals, relieves the repression exerted on the p53 pathway and predisposes cells to apoptosis^[19]. This may be the case for other oncogenes such as the pituitary tumor transforming gene 1 (PTTG1)^[20].

Another example is the catalytic subunit of Telomerase (Telomerase Reverse Transcriptase, TERT). Since telomerase function is necessary to maintain telomere length in each cell division, cancer cells undergoing unrestricted cell proliferation present activation of TERT expression^[21]. Therefore, inhibition of TERT is hypothesised to cause inhibition of cell growth after several divisions, when telomeric repeats finally run out. However, efficient inhibition of telomerase expression is able to induce apoptosis in a few days, and this is irrespective of its telomere-lengthening function^[22]. New data indicate that telomerase is also necessary to protect chromosome ends from being recognized as DNA disruptions, which would trigger apoptosis^[23].

Different methods are used to inhibit expression of oncogenes. One of them is based on the transfer of antisense nucleotides, artificial sequences complementary to the mRNA corresponding to the gene whose inhibition is attempted^[24]. These can be short sequences (antisense oligonucleotides, ASO), or the full cDNA. Several mechanisms account for the blocking of gene expression, with the most widely spread and studied being the degradation of RNA-DNA hybrids by cell nucleases such as RNase H. A more recent approach is RNA interference, another posttranscriptional gene silencing mechanism based on the production of double-stranded stretches of RNA complementary to the target mRNA^[25]. Using the endogenous cell machinery, the double-stranded RNA is processed into 21 to 23-nucleotide short interfering RNAs (siRNAs) that recognize the cognate mRNA and trigger its degradation. Alternatively, the siRNAs can be transfected directly. In the "triple helix" strategy, the inhibitory oligonucleotides (triplex-forming oligonucleotides, TFOs) are targeted to the cellular double-stranded DNA^[26]. They interact with polypurine-polypyrimidine sequences in the minor or major groove of genomic DNA and block gene expression at different levels depending on the localization of the complementary sequence. They could be potentially used not only for gene expression modification, but also in gene correction strategies^[27]. Finally, the expression of secreted or intracellular antibody-based molecules has been proposed to block the function of oncogenes^[28,29].

In the case of HCC, the inhibition of several genes has shown potential antitumor effect. Most reports provide

proof of concept showing growth inhibition or induction of apoptosis using HCC-derived cell lines in cell culture. The *in vivo* studies performed in animal models show growth retardation in tumors, especially when cancer cells are transfected *ex vivo*, but complete eradication is difficult when *in vivo* gene therapy is tested on pre-existing tumors.

Since telomerase and Wnt pathway activation are frequently associated with HCC, different approaches including antisense molecules and siRNA have been used to inhibit them^[30-32]. Antisense technology was also used against FGF-2^[33], VEGF^[34] and COX-2 genes^[35]. The triplex helix approach showed similar results as antisense technology for the inhibition of IGF-I and induction of apoptosis in HCC cells^[27]. When the cells were injected into mice, an immune-mediated antitumor protection was observed. The inhibition of PTTG1 and urokinase-type plasminogen activator (u-PA) has been accomplished using siRNA on HCC cells^[20,36]. The p28-GANK oncoprotein, which induces hyperphosphorylation and increases degradation of pRB was found to be overexpressed in the majority of HCCs^[37]. The repeated administration of an adenoviral vector that induces the production of siRNA against p28-GANK caused a dramatic decrease in the growth of human HCC xenografts in nude mice^[38]. This shows that the continuous inhibition of an oncogene may have a strong impact on the progression of tumors. The clinical application of this approach is challenging, because highly efficient long-term expression vectors will be needed instead of first generation adenoviruses.

GENE-DIRECTED ENZYME/PRO-DRUG THERAPY (GDEPT)

This approach is based on the transfer of exogenous genes that convert a non-toxic pro-drug into a cytotoxic metabolite in cancer cells^[39]. Once the pro-drug is administered systemically, transduced cells expressing the converting enzyme die and, in some cases, provoke the destruction of surrounding cells (bystander effect). Unlike other gene therapy strategies, GDEPT lacks intrinsic tumor specificity, and relies on tumor targeting at the levels of cell transfer (depending on the vectors and the route of administration) and gene expression (depending on tumor-specific promoters)^[40]. The efficacy of a GDEPT system is highly influenced by the extent of the bystander effect, because the fraction of transduced cells in a tumor is generally low with current gene therapy vectors^[41].

The thymidine kinase gene from HSV-1 (HSV-TK) used in conjunction with the pro-drug ganciclovir (GCV) was the earliest and most used GDEPT system applied to HCC and other cancers^[42]. It has shown significant antitumor effect in relevant animal models of HCC, such as carcinogen-induced HCC in rats^[43]. HSV-TK converts ganciclovir into the monophosphate intermediate that is subsequently transformed into the triphosphate form by cellular enzymes. This is a highly polar molecule that cannot diffuse outside the cell. The bystander effect of this system has been explained by gap junction transfer of the toxic metabolite and phagocytosis of neighbouring cells^[44], but this local effect is weak compared to other

GDEPT modalities. The fusion of TK with the VP22 protein can amplify the effect by transferring the enzyme to surrounding cells^[45]. Ganciclovir-triphosphate is incorporated into the DNA and causes apoptosis in a cell cycle-dependent manner, but it can cause mitochondrial toxicity in normal hepatocytes if the expression of HSV-TK is not restricted to HCC cells^[46,47]. Apart from the therapeutic purpose, HSV-TK can be considered a reporter gene for PET analysis. It has been successfully used to visualize transduction of HCC with adenoviral vectors in humans^[48]. So far, the good antitumor efficacy of the HSV-TK system observed in different animal models of HCC has not been demonstrated in the clinical setting^[42]. Clinical and pre-clinical studies performed on other cancers suggest that HSV-TK can act as an immunogen that cooperates in the establishment of a systemic or at least local response against the tumors^[49,50]. Nevertheless, combination with other therapies will be needed. In pre-clinical studies the radiation-inducible Egr-1 promoter was used to control the expression of HSV-TK in combination with radioisotopes (I^{131} lipiodol)^[51]. Thus, the expression of HSV-TK was stimulated by the internal radiation, and the antitumor effect of both treatments was synergistic.

The yeast Cytosine Deaminase converts the antifungal drug 5-fluorocytosine (5-FC) into the cytotoxic thymidylate synthetase inhibitor 5-fluorouracyl (5-FU)^[52]. This metabolite can diffuse locally and cause a wider bystander effect than phosphorylated ganciclovir, but the cytotoxicity is also cell cycle-dependent. The system has been used in animal models of primary and metastatic liver cancer with good results^[53,54]. The efficacy of 5-FU on HCC patients is very low, but this strategy could achieve high local concentrations of the drug. In this context, toxicity in normal liver should be carefully evaluated. In addition, the conversion of 5-FC to 5-FU by the cytosine deaminase present in habitual enterobacteria can contribute to toxicity^[55].

Other GDEPT approaches generate very potent DNA cross-linking agents whose effects are largely cell cycle-independent. These include the cytochrome P450/cyclophosphamide^[56] and the Nitroreductase/dinitrobenzamide CB systems. Palmer *et al*^[57] reported that the intratumor administration of a first generation adenoviral vector expressing Nitroreductase in HCC patients is safe and feasible. Transgene expression was dose-dependent and is supposed to be clinically relevant, although no pro-drug was administered to patients in this study. Strong immune responses against the vector and the therapeutic gene were observed, indicating that re-administration of the treatment may not be beneficial. Assessment of the antitumor effect and toxicity of this approach in patients receiving the pro-drug requires new clinical trials.

An approach closely related to GDEPT consists on the delivery of the sodium iodide symporter (NIS) gene to cancer cells^[58]. Since NIS is necessary for the internalization of I^{131} in the cell, a higher dose is accumulated in cells expressing NIS, as happens in thyrocytes, resulting in cell cycle blockade and death. Using this method, the extent and location of gene transfer can be detected by tomography. An adenovirus vector

expressing NIS under the control of the CMV promoter has been used for the treatment of HCC in a model of chemically induced tumors in rats^[59]. After injection of the vector in pre-existing nodules, specific accumulation of I^{131} and significant reduction in tumor volume was observed.

TARGETED EXPRESSION OF CYTOTOXIC/PRO-APOPTOTIC GENES

This strategy is based on the selective transfer of genes that will cause the destruction of the cancer cells by different mechanisms. The concept is similar to GDEPT, but in this case the effect does not depend on any exogenous drugs. This can be an advantage in some circumstances, but on the other hand it lacks the possibility of pharmacologically modulating the cytotoxicity. This means that the system relies mostly on the targeting of gene transfer and expression into cancer cells, using specific surface ligands or promoters. The promoters for α -fetoprotein (AFP) and TERT have been used to control the expression or the diphtheria toxin fragment A and other cytotoxic genes in HCC cells^[60,61], but the toxicity of these treatments in relevant animal models is unclear.

Alternatively, the mechanism of action of the lethal gene can provide some tumor specificity. This is the case for TNF-related apoptosis inducing ligand (TRAIL). Unlike other members of the TNF ligand family, such as FASL and TNF- α , TRAIL induces apoptosis preferentially on cancer cells and may have reduced hepatotoxicity^[62]. The extracellular domain of TRAIL works as a soluble cytokine (sTRAIL) and induces apoptosis of cancer cells at distant locations from the producing cell. In fact, an AAV vector expressing sTRAIL fused with a human insulin signal peptide has shown potent antitumor effect on subcutaneous liver cancer xenografts after oral or intraperitoneal administration of the vector^[63]. This systemic effect was achieved without significant liver toxicity. Other vectors developed for the expression of TRAIL include first generation and oncolytic adenoviruses with enhanced infectivity in cancer cells^[64,65]. Interestingly, the adenoviral E1A protein sensitizes cells to TRAIL-induced apoptosis^[66].

IMMUNOGENE THERAPY

The transfer of genes with the aim to elicit an immune response against tumors is one of the most extensively used strategies in the field of cancer gene therapy. It is based on the observation that cancer cells modify their characteristics and their environment in order to avoid being detected and rejected. If this can be reversed, the specificity and systemic nature of the immune system offers the possibility of controlling the primary tumor and block its dissemination, which is the ultimate goal of all oncologic treatments. The wide repertoire of immunogene therapy approaches can be grouped as follows.

Expression of immunomodulatory cytokines

Cytokines are key mediators in the function of the immune system. They have been extensively used to

stimulate the immune response against tumors, including interleukin 2^[67], 7^[68], 12^[69], 15^[70], 18^[71], 21^[72], 23^[73] and 24^[74]; interferon α ^[75], β ^[76], and γ ^[77]; tumor necrosis factor α ^[78]; granulocyte-macrophage colony stimulating factor (GM-CSF)^[79], and others. Their effects on different cell components of the immune system and their influence on the expression of endogenous factors are extremely complex. Most of these cytokines do not have an intrinsic tumor-specific effect, but they may enhance the precarious immune response against tumors if the dose, location and timing are carefully controlled. For example, interleukin-12 (IL12) promotes a T-helper cell type 1 (Th1) response with activation of cytotoxic T lymphocytes and natural killer cells (NK)^[80], together with an antiangiogenic effect^[81,82]. These effects are largely dependent on the induction of IFN- γ . The systemic administration of recombinant IL12 showed potential antitumor effects in humans^[83], but severe toxicity was observed^[84] and this modality of treatment was discarded. The use of gene therapy vectors enables the localization of IL12 expression to the tumor, especially if vectors with liver tropism such as those derived from adenovirus are used^[85]. The antitumor effect of this strategy on different animal models of HCC has been demonstrated by several groups^[86,87]. Tumor eradication and immunologic protection against relapse is achieved in a significant proportion of cases, including implanted tumors in syngenic animals and chemically-induced HCC in rats. These results led to a phase I clinical trial that demonstrated the safety and feasibility of intratumor injection of a first generation adenoviral vector expressing IL12 in primary and metastatic liver cancer patients^[88]. Using these vectors, the expression of IL12 was very low and transient. No complete responses were observed, but patients with HCC had a better outcome than other histological groups in this trial. Based on these results, improvements in the vectors are being investigated. The use of high-capacity adenoviral vectors carrying a liver-specific inducible system for the expression of IL12 allows the long-term expression of the cytokine in response to the inducer mifepristone. Using this vector, the levels and duration of cytokine expression can be modulated to achieve antitumor effect and avoid toxicity^[89]. Further improvement can be achieved by using a version of IL12 in which the p35 and p40 subunits are fused in a single protein using a short linker peptide^[90]. Experiments performed in rats bearing HCC indicate that the single chain IL12 is about 1000 times more potent than the native protein when an equivalent adenoviral vector is used to deliver the gene intratumorally^[87].

Other cytokines that deserve special attention are TNF- α and IL24 (also known as mda-7). These molecules have shown antitumor effect on animal models of HCC^[78,91], and ongoing clinical trials suggest the potential therapeutic effects on other malignancies in humans^[92,93]. IL24 is especially promising, because apart from its immune-regulatory activities it induces apoptosis preferentially in cancer cells^[94].

Taking into account the natural mechanism of immune response activation, pro-inflammatory cytokines and co-stimulatory signals should be combined to achieve an effective response and avoid anergy. It is possible that

the accessory signals are already present in the tumor, but there is evidence of enhanced antitumor effect when IL12 is transferred together with 41BB agonists^[95] or B7.1^[96] in animal models of HCC. The intratumoral injection of an adenoviral vector expressing CD40L achieved tumor eradication on a significant proportion of pre-existing HCC in a rat model^[97]. This molecule is normally expressed on activated T cells and interacts with CD40 on the surface of antigen-presenting cells.

A combination of different cytokines may be more effective and less toxic than the expression of a single cytokine at high levels. The injection of adenoviral vectors expressing IL12 and IP-10 (interferon- γ inducible protein-1) exerted a synergistic antitumor effect in a murine model of colon cancer when both molecules were expressed locally^[98]. This is in agreement with the "attraction and activation hypothesis", in which colocalization of immunostimulatory (IL12) and chemoattractant factors (IP10) is needed. Some pre-clinical data indicate that IL15 can increase the antitumor effect IL12 on HCC models^[99]. Interestingly, this could happen in the absence of IFN- γ function. Other combinations proposed for the treatment of HCC include IL12+GM-CSF^[100], IL12+MIP3 α ^[101], and IL21+IL15^[72]. An alternative to co-expression of individual cytokines is the construction of fusion proteins. You *et al.* used a retroviral vector to deliver an IL2/IL12 fusion gene and demonstrated enhanced survival of tumor-bearing rats compared with rats treated with the individual cytokines^[102]. The antitumor effect of cytokines can be enhanced by other gene therapy approaches like GDEPT using HSV-TK, as demonstrated by several groups that employed adenoviral or retroviral vectors for gene delivery in HCC models^[103,104].

Vaccination with tumor antigens and genetically modified cells

The transfer of genes encoding tumor-specific antigens such as AFP has been used with the aim to break the immune tolerance against HCC^[105]. The pre-clinical efficacy of this approach depends on the particular animal model employed^[106], suggesting that high variability could be expected in patients. A different approach consists on the administration of activated effector or antigen-loaded presenting cells to fight cancer. The efficacy of these cells can be increased if they are manipulated genetically to express antigens, cytokines or co-stimulatory molecules (*ex vivo* gene therapy). Syngenic fibroblasts or cancer cells expressing IL12^[107] or IL2 plus B7^[108] can trigger an immune response against HCC in murine models. However, the use of cancer cells as a source of antigens and cytokines poses obvious technical difficulties in the clinical setting. An attractive alternative is the use of autologous dendritic cells (DC), professional antigen presenting cells that express the co-stimulatory molecules (CD80, MHC class I and II, *etc.*) necessary for efficient activation of effector cells. DCs expressing AFP^[109], cytokines^[110] or co-stimulatory molecules^[111] have been successfully used in animal models of HCC and gastrointestinal cancer^[112]. These results encouraged the initiation of a phase I clinical trial in which DCs expressing IL12 after *ex-vivo* infection with an adenoviral

vector were injected into the tumor mass^[113]. However, it was demonstrated that the cells were unable to migrate to lymph nodes because they were sequestered into the tumor by local factors^[114], preventing an efficient activation of effector cells and the establishment of relevant antitumor immune responses.

Adoptive cell therapy consists on the infusion of autologous T cells or killer cells that have been expanded and activated *in vitro*. In animal models, it has been demonstrated that T cell expansion occurs *in vivo* in tumor-bearing mice that were treated with IL12^[115]. The infusion of these cells has antitumor effect on recipient mice, in synergy with *in vivo* gene therapy by an adenoviral vector expressing IL12. This suggests that immunogene therapy can be used in combination with adoptive T-cell therapy in order to increase the efficacy observed in clinical trials that used either strategy alone.

An important aspect that is becoming more relevant in recent years is the inhibition of regulatory signals that control the duration and intensity of the immune response, because this could enhance the efficacy of anticancer immunotherapy. For instance, the blockade of the B7-H1/PD-1 pathway by soluble PD-1 expression improved the immune response against an implanted HCC in mice^[116].

ANTI-ANGIOGENIC GENE THERAPY

The realization that tumor growth requires intense neo-vascularization is the basis for a series of approaches aimed to specifically block the cancer-induced formation of new vessels^[117]. Anti-angiogenic factors such as endostatin have been identified and have demonstrated the ability to inhibit tumor growth *in vivo*^[118,119]. This strategy is supposed to be safe because it does not affect the mature vessels of normal tissues. Since HCC is known to be much vascularized, antiangiogenic therapies may have a strong therapeutic benefit, probably in combination with other standard or experimental treatments. Gene therapy may play an important role in this field, because anti-angiogenic factors need to be delivered for long period of times to control the progression of tumors. In fact, an adenoviral vector carrying the endostatin cDNA was more effective than the direct injection of the protein^[120]. The combination of endostatin delivered by an AAV vector and chemotherapy (etoposide) achieved antitumor effect on metastatic liver cancer in mice^[121]. Of note, several strains of bacteria have been engineered as vectors to deliver endostatin into liver tumors. Bifidobacterium longum administered orally increased the survival of tumor-bearing mice^[122], whereas attenuated Salmonella choleraesuis accumulates in hypoxic tissues and has shown antitumor effect after intraperitoneal administration^[123].

Other anti-angiogenic approaches are focused on blocking the VEGF receptor, which is an important mediator of angiogenesis. This can be achieved by expressing the soluble form of VEGF receptor (KDR/Flk-1), which sequesters VEGF^[124]. The same approach has been used to block the endothelium-specific receptor Tie2, which affects direct tumor growth and neovascularization^[125]. The Pigment Epithelium Derived Factor (PEDF) has been recently discovered as an anti-

angiogenic protein expressed in normal liver^[126] that is downregulated in HCC patients, suggesting a possible role in tumor progression. The transfer of PEDF has antitumor effects in a murine model of HCC^[127]. NK4 is a fragment of the Hepatocyte Growth Factor (HGF) that acts as a HGF antagonist and blocks angiogenesis. The intrasplenic administration of an adenoviral vector expressing a secreted form of NK4 caused reduction in the vascularization and growth of pancreatic metastasis in the liver of mice^[128]. Finally, it should be mentioned that the inhibition of angiogenesis may be one of the most important mechanisms by which IL12 exerts its antitumor effect^[129].

ONCOLYTIC VIRUSES

Using the cytopathic effect of certain viruses to destroy cancer cells is an old idea, but the advances in viral vector design and production have renewed interest in the field of virotherapy. The objective is to obtain a virus that replicates and preferentially kills cancer cells, leaving the surrounding normal tissues relatively intact^[130]. This property is intrinsic to some viruses. For instance, Vesicular Stomatitis Virus (VSV), Measles Virus (MV) and Newcastle Disease Virus (NDV) are very sensitive to the inhibitory effects of IFN and replicate only in cancer cells that have developed mechanisms to counteract IFN pathways. Other viruses like reovirus replicate better in cells that present activation of the Ras oncogene^[131].

On the other hand, other viruses such as Adenovirus or HSV can be genetically modified to make their replication cancer-specific. One of the methods to achieve cancer specificity is the deletion of viral functions necessary for replication in normal cells, but not in cancer cells. For instance, the adenoviral protein E1A blocks pRB in the cell to force activation of the cell cycle, whereas E1B 55K blocks p53 to inhibit apoptosis at early times. Since both p53 and pRB pathways are commonly altered in cancer cells, adenoviruses lacking these functions will replicate preferentially in tumors^[132,133]. Another method to restrict the replication of viruses is to use tumor-specific promoters to control the transcription of viral genes important for replication, such as E1A and E4 for adenovirus^[134]. Parallel strategies have been used to achieve oncolytic herpes viruses^[135,136]. The control of the γ 134.5 gene expression determines the efficacy of HSV-1 replication in different cells, and the deletion of the ribonucleotide reductase function attenuates the virus in normal cells^[137].

An important property of oncolytic adenoviruses is the possibility of accommodating therapeutic genes and the ability to act as gene therapy vectors with the advantage of tumor-specific amplification of gene expression^[138]. These genes code for pro-drug converting enzymes, immunostimulatory cytokines or pro-apoptotic proteins that enhance the oncolysis and/or achieve a systemic effect.

The mutant dl1520 adenovirus (also called ONYX-015 or CI-1042 later on) was described in 1996 as the first oncolytic adenovirus^[139]. It contains a deletion in the E1B 55K gene that achieves preferential replication in

cancer cells by different mechanisms^[140]. Although recent advances have yielded viruses with improved potency and specificity, the experience accumulated with ONYX-015 in the laboratory and in the clinic has been extremely useful for the advance of the field. The virus has shown partial antitumor effect on murine models of HCC^[141], and clinical trials for other cancers indicate a potential benefit when used in combination with chemotherapy^[142]. In the case of liver cancer, a clinical trial on HCC patients showed no evident antitumor effect. The first dose of ONYX-015 was administered intravenously and then by direct ultrasound-guided intratumoral injection on d 2, 15, 16, 29 and 30^[143]. The rationale of this regime was to elicit an immune response against the virus that causes a local reaction in the tumoral site. In a separate phase II trial in patients with metastatic colorectal cancer the virus was administered intravenously, and only transient stabilization of the disease could be observed in some cases^[144]. When the virus was administered intratumorally in a different clinical trial for hepatobiliary tumors, transient reduction of tumor markers in serum (CEA, AC19-9 or AFP) was observed in 50% patients, although radiological responses were less than 10%^[145]. These results support the notion that ONYX-015 has limited therapeutic effect as monotherapy on HCC patients, especially if systemic routes are used. When the virus was administered intravenously in combination with 5-Fluorouracil and leucovorin in patients with liver metastases of gastrointestinal cancers, 25% of cases presented partial or minor (< 50%) radiological responses, with good tolerance and evidence of adenovirus replication in tumors^[146]. An independent trial in patients who had failed previous treatment with 5-FU suggested increased survival when the virus was injected in the hepatic artery in combination with 5-FU^[147]. Interestingly, an early radiological increase in tumor volume was attributed to virus-induced necrosis rather than tumor progression in several patients. This should be taken into account in order to evaluate efficacy and avoid removal of responding patients in clinical trials. To this end, PET can be a more reliable technique. Now clinical trials with a virus similar to ONYX-015 (H101) are being conducted in China. A phase III trial in squamous cell cancer patients showed increased response rate in combination with chemotherapy, and the virus has recently obtained approval in that country^[148].

Other oncolytic adenoviruses have been developed, and show promising results (usually better than ONYX-015) in animal models of HCC. However, their performance in clinical trials has not been tested so far. The AFP promoter was used to control the expression of the E1A viral gene, with or without E1B 55K deletion, and this achieved preferential replication in AFP-producing HCC cells^[149,150]. The same effect is observed in metastatic gastrointestinal cancer using a virus controlled by the CEA promoter^[151]. A broader cancer spectrum is achieved when other tumor-specific promoters such as human TERT^[152,153] and E2F-1^[154] are used. The efficacy of these agents can be increased if they are adapted as gene therapy vectors for therapeutic genes ("armed" viruses), because viral oncolysis usually cooperates with the effect of the gene. Oncolytic adenoviruses expressing GM-

CSF^[155], TRAIL^[65,156], Smac^[157], Cytosine Deaminase^[158] and endostatin^[159] have demonstrated better performance than the previous versions.

The field of virotherapy has been enriched by the incorporation of oncolytic agents derived from different viruses, which may solve some of the limitations observed with adenovirus. For instance, HSV-1 exerts a potent oncolytic effect and its large genome can accommodate different exogenous genes, apart from the endogenous TK^[160]. The complex genome of HSV-1 allows multiple modifications that can be exploited to achieve tumor specificity. The G207 mutant contains a disruption in the UL39 gene that eliminates the ribonucleotide reductase function and determines preferential replication in cancer cells^[161]. It is attenuated by deletion of a single copy of the ICP6 gene and both copies of the γ 134.5 neurovirulence gene, but efficient elimination of HCC cells has been reported^[162]. The NV1020 virus harbours a deletion over the joint region of the genome, but retains the ICP6 gene and one copy of γ 134.5^[163]. A clinical trial using this virus is ongoing for patients with gastrointestinal cancer metastatic to the liver. The rRp450 HSV-1 variant carries the cytochrome p450 gene as a pro-drug converting enzyme^[164]. This virus has shown promising antitumor effect on HCC models, although complete eradication of metastatic liver cancer was not observed after single or multiple intraportal administrations. In addition, significant antitumor effect has been obtained in liver cancer models using herpes virus expressing Cytosine deaminase^[165] or IL12^[166].

VSV-derived viruses are emerging as a new class of oncolytic agents. A single injection of a recombinant VSV virus into the hepatic artery increased the survival of rats bearing multifocal HCC, and multiple doses achieved long term survival and tumor eradication in nearly 20% of the animals^[167]. This strategy is being investigated in human patients. Interestingly, experiments performed in rats indicate that a prophylactic treatment with IFN- α reduces the toxicity of the virus on normal tissues and elevates its therapeutic index^[168].

CONCLUSION

The treatment of hepatic malignancies (both primary tumors and metastatic cancer of the liver) remains a challenge that needs new approaches. Gene therapy is an experimental discipline in continuous evolution that offers interesting opportunities for the treatment of liver cancer. Following early excitement about gene therapy possibilities, the field soon realized its limitations and is now systematically addressing fundamental issues to solve them. The transfer of genes to the majority of cancer cells is still unrealistic for solid tumors, even with the best vectors available to date. Immunogene therapy approaches try to circumvent this limitation and extend the antitumor effect to distant metastases. Pre-clinical studies have validated the concept, but at the same time the results in animal models reveal that the efficacy of immunotherapy is very limited in advanced liver cancer. Hence, it is not surprising that the results of early phase clinical trials are apparently deceiving. Oncolytic adenoviruses were envisioned as autonomous

therapeutic agents that would seek and destroy cancer cells, amplifying the initial load until the tumor is eradicated. Now we know that they find important physical barriers that limit their distribution inside the tumor. Moreover, the immune system will control the spread of the viruses in a few days and neutralize further administrations, leaving a narrow time frame for them to display their oncolytic activity. An additional obstacle for the clinical application of most gene therapy approaches is the cost and technical difficulties of large scale production of the vectors.

Despite all these difficulties, gene therapy may play an important role as an adjuvant to other standard or experimental treatments against liver cancer in the near future. There is evidence that different gene therapy approaches like GDEPT or oncolytic viruses have synergistic effects when combined with chemotherapy or radiotherapy. The different mechanisms of action favour these combinations and may prevent the development of resistance to the treatment. As the knowledge of tumor immunology advances, more rational immunogene therapy approaches are designed. In addition, the improvement of invasive techniques for locoregional treatment of HCC can be used to deliver gene therapy vectors inside the tumor, increasing their safety and efficacy.

In summary, gene therapy will improve the management of liver cancer patients in the future, probably as part of an individualized multimodal therapy. This will require close collaboration and a continuous flow of information between basic, applied researchers and health care professionals.

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