Review

Gene therapy of hepatocarcinoma: a long way from the concept to the therapeutical impact

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Hepatocellular carcinoma (HCC), the most prevalent histological form of primary liver cancer is one of the most frequent cancer worldwide. This pathology still requires the development of new therapeutical approaches. Gene therapy strategies focusing on the genetic manipulation of accessory cells involved in the immune reaction against cancer cells, or on the direct transduction of tumor cells with transgenes able to "suicide" cancer cells have been largely developed for more than ten years.

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Primary liver cancer is the sixth most frequent cancer worldwide. Hepatocellular carcinoma (HCC), the most prevalent histological form, is usually associated with liver cirrhosis and/or chronic viral hepatitis.^{1–3} Its incidence increases in North American and Western European countries and in Japan.^{4,5} Liver transplantation, which allows the treatment of both malignant pathology and hepatic deregulation, is now considered the most satisfying therapeutical approach; however, considering the difficulty to obtain transplantable grafts and the occurrence of relapse, this strategy is restricted to patients exhibiting less than three visible and small-size nodules.^{6–10} Large-size tumors are eliminated by a surgical procedure that does not treat intrinsic hepatic deregulation and that is associated with a high risk of relapse.^{11,12} Alternative strategies such as arterial chemoembolization, percutaneous ethanol injection or radio-frequency ablation have been tested without showing any improvement in the median survival of patients. Thus, the treatment of HCC still requires the development of new therapeutical approaches and could largely benefit from gene therapy strategies.^{9,13–16} Gene therapy relies on the transfer into a selected cell population of a gene coding for a therapeutic protein whose expression is placed under the control of regulatory elements (promoters and enhancers) that enable the transcription of the coding sequence. Gene therapy strategies focus on the genetic manipulation of accessory cells, such as dendritic cells or

immunoreactive T lymphocytes to control the immune reaction against cancer cells, or on the direct transduction of tumor cells with transgenes able to "suicide" cancer cells mainly using apoptosis-related mechanisms.

A complex context

In the normal liver, the fenestration of endothelial cells and the absence of basal membrane surrounding endothelial sinusoid cells allow the vectors to easily diffuse to the hepatocytes following injection into the peripheral veins or following intraportal injection. Conversely to normal hepatocytes, tumoral cells have been shown to be more difficult to access. First, HCC blood supply depends preferentially upon the hepatic artery; next, the so-called capillarization phenomenon (disappearance of endothelial cells fenestrae and development of a basal membrane) associated to perivascular fibrosis and extracellular matrix deposition may dramatically limit the accessibility of tumoral cells.^{17–19} Second, we observed a massive diffusion of adenoviral vectors to nontumoral liver tissue after a direct intratumoral injection of the adenoviral preparation in the woodchuck HCC animal model, which could be due to vascular anastomosis between tumor vasculature and the portal vein tract.²⁰ The possibility to modulate the blood–tumor barrier, which limits gene transfer with vasoactive compounds, has been tested and proved to be of value in gene therapy of HCC using adenoviral vectors.²¹ In addition, the used animal models do not mimic the multiparameter context of chronic liver disease or the cirrhosis which favor the emergence of HCC. Human HCC are frequently multifocal and are associated with other dysplastic areas within the

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nontumoral liver, which could be seen as a preneoplastic state. Accordingly, the widely used animal models do not exhibit the long-term liver insufficiency that can be associated with HCC and can make some cytotoxic treatments dangerous.^{9,18}

Experimental models

The expression of the transgene in the target cell *in vitro* is a classical prerequisite to ascertain the possibility to transduce the target cells with the selected vector, the expression of the transgene and the potential biological activity of the protein. Numerous experiments have been performed with cell lines derived from HCC such as HUH7, HepG2, etc., but a cell line in a dish does not mimic a tumor mass *in vivo*. HCC cell lines actively proliferate conversely to primary tumor cells *in vivo* and exhibit a homogenous differentiation status conversely to HCC cells. In addition, this strategy does not take into account the tridimensional structure of the tumor and the interactions with neighboring tissues.

The implantation of xenogenic cells to develop a tumor in immunodeficient animals can be considered as a potential alternative to *in vitro* approaches even if this strategy remains highly artificial. In such models, pre-established HCC cell lines are subcutaneously or orthotopically reinjected to generate tumors. The possibility to inject *in vivo* the viral vector and the therapeutical impact of the strategy can be assessed, but the manipulation of implanted tumors does not feature the difficulties of approaching the treatment of native tumors in the liver and the possibility to elicit an immune response against cancer cells cannot be tested with immunodeficient animals. In addition, injection of gene therapy products into subcutaneous tumors does not allow the analysis of toxic effects deleterious for the liver. The development of syngenic models with the implantation of rat HCC cell lines into Buffalo rats or the injection of Hepal-6 cells in C57L/J mice^{22,23} has been considered. At least, such models should allow the analysis of the impact of the immune system on the elimination of gene transfer vectors.

Although naturally occurring HCC has been described in transgenic mice that overexpress the Simian Virus 40 (SV40) large T antigen into the liver, virally or chemically induced HCC in the animal model can be considered as the closest model to human pathology.²⁴ For example, diethylnitrosamine (DEN)-induced HCC in the rat share several features with human HCC with tumors that arise from dysplastic areas and exhibit a predominant arterial blood supply.^{17,25,26} Although the woodchuck does not exhibit liver cirrhosis following infection with the Woodchuck Hepatitis Virus (WHV), WHV-induced HCC in the woodchuck model showed some close similarities with Human Hepatitis B virus related HCC in humans since almost all the WHV-infected woodchucks that develop chronic hepatitis have been shown to develop multifocal HCC.^{27,28} The large size of the

woodchuck model facilitates intratumoral or intra-arterial injection of gene therapy products. However, it should be noted that this model is very expensive and, according to its size, requires the use of large amounts of gene therapy products.

Gene transfer vectors for HCC target cells

Gene transfer relies on the use of a genetically engineered vector, of viral or nonviral origin, that promotes the transfer of the transgene expression unit into the intracellular compartment and allows its transient or stable expression. Considering transduction efficiency and expression of the transgene are two key parameters to be considered when promoting a therapeutical effect with genetically manipulated cells, numerous works still focus on the selection and/or the design of the most efficient gene transfer strategy.

Viral vectors

Viral vectors are now considered as the most powerful tool to transfer and express a transgene of interest in a target cell. The construction of viral vectors relies on the replacement of viral sequences encoding for proteins involved in the replication cycle with the expression unit of interest. Replication-defective viral vectors, produced with cell lines engineered to synthesize the *trans*-active proteins that are absent from the viral vector genome, can transfer the selected expression unit in the target cell without producing new viral particles, thus preventing further dissemination of the viral vector. The feasibility of strategies using replication-competent viral vectors that are able to replicate only in cancer cells, an approach that would allow the spread of the viral vector, has been questioned with adenoviral vectors in the treatment of colorectal carcinoma metastases localized in the liver, but remains to be developed for the treatment of HCC.²⁹

Adenoviral vectors: a classical but controversial choice. Adenoviral vectors usually derive from type 2 or type 5 adenoviruses, a nonenveloped DNA virus, by replacement of the E1A region of the adenovirus genome with the transgene of interest placed under the control of an exogenous promoter.³⁰ Adenoviral vectors have been shown to be very efficient in transducing *in vitro* and *in vivo* numerous cell types regardless of the replication status of the target cell.³¹ Highly concentrated adenoviral vector-containing stocks can be produced with up to 10¹³ viral particles per milliliter that facilitates *in vivo* experiment. The genome of the viral vector does not integrate into the DNA of the target cells and is retained under an episomal configuration that does not allow the maintenance of expression of the transgene in actively proliferating cells. Assuming an efficient transduction and a rapid therapeutical effect of the protein encoded by the viral vector, this feature does not *a priori* preclude the use of adenoviral vectors for cancer gene therapy. The injection of adenoviral vectors has been shown to elicit strong immune responses against viral particles or

transduced cells.³¹ Although such an immune response against tumor cells can be of interest, the potential rapid elimination of viral particles or the difficulties to reiterate the injection of viral particles of the same serotype sometimes makes difficult the use of adenoviral vectors.³¹ In addition, considering that adenoviral vectors injected *in vivo* can transduce nonselected cells, a strong immune response against normal cells such as normal hepatocytes could be very deleterious for the patient. One of the main problems with adenoviral vectors is the strong liver toxicity due to an acute inflammatory response involving the release of inflammatory cytokines such as Interleukin-6, Interleukin-8 and Tumor Necrosis Factor alpha making critical the injection of large amounts of viral particles.³² Conversely, intratumoral, intra-arterial and intravenous administrations of low doses seem well tolerated.^{33,34}

Several strategies have been approached to circumvent these problems. Adenoviral vectors devoid of viral *trans*-active sequences, the so-called gutless vectors, have been constructed but these vectors remain difficult to produce.³⁵ Overexpression of viral proteins involved in the induction of a tolerance of the immune system against adenoviruses, such as proteins encoded by the E3 region, has been tested to prevent antiviral humoral and cellular immune responses.³⁶

Numerous works report the use of adenoviral vectors for gene therapy of HCC. Adenoviral vectors transduce efficiently HCC cells *in vitro* and *in vivo* as shown by the transfer of therapeutic or marker genes in DEN-induced HCC in the rat model or in WHV-induced HCC in the woodchuck model.^{20,26} *In vivo* gene transfer efficiency depends on the route of administration and the size of the tumors. Small-size tumors and dysplastic nodules in rat DEN-induced HCC can be efficiently transduced when using the arterial route, whereas only direct intratumoral administration allows efficient transduction of HCC in large-size tumors.²⁶ There have been numerous efforts to restrict the expression of the transgene to HCC using specific promoters.³⁷⁻⁴⁰

Adeno-associated virus (AAV)-derived vectors: an outsider to be stimulated. The elimination of all the coding regions of the AAV type 2 allows the generation of AAV vectors able to transfer up to a 4.8 kb long expression unit. AAV vectors can efficiently transduce HCC cell lines and normal hepatocytes *in vitro* and *in vivo*.^{20,26} However, transduction with AAV vectors often requires a chemical, physical (irradiation) or a viral (adenovirus infection) costimulation, which makes complex the use of these vectors in a clinical context.⁴¹ In addition, the small size of the insert, up to 4.8 kb, prevents the use of large genes or expression units exhibiting tissue-specific promoters with large regulatory sequences. The effectiveness of AAV vectors to genetically modify HCC in models with implanted tumors has already been tested with the Herpes Simplex Virus Thymidine Kinase Gene (HSV-TK) gene placed under the control of a ubiquitous promoter or the AFP promoter.⁴²

Murine leukemia virus (MLV)-derived retroviral vector: waiting for the mitosis. MLV derived retroviral vectors can integrate the transgene of interest into the genome of the target cells that allows the vertical transmission of the therapeutic information. Most of the vectors used in a clinical context derive from the Moloney murine leukemia virus. The gag, pol and env coding sequences of the native retrovirus have been eliminated to generate MLV-derived vectors and the transgene of interest can be placed under the control of regulatory elements localized in viral *cis*-active elements called long terminal repeat (LTR), exhibiting eucaryotic promoter elements and involved in the replication cycle of retroviruses, or placed under the control of an exogenous promoter. Stable or transient cell lines engineered to constitutively produce the retroviral gag, pol and env proteins and the gene transfer vector are used to synthesize viral particles. The emergence of replication-competent viruses (RCV) due to recombination between the gene transfer vector, endogenous retroviral sequences in producing cell lines and/or elements present in the gag, pol and env expression units has been considered as a major safety concern. Numerous strategies such as deconstruction of the viral backbone, development of self-inactivating vectors exhibiting deletion of major *cis*-active elements after transduction, use of cell lines shown to not favor recombination between exogenous and endogenous retroviral elements, development of a sensitive assay to detect RCV, etc., have been successfully approached to strongly secure the use of retroviral vectors.⁴³

Several problems can be encountered when using retroviral vectors. Although some efficient producing cell lines have been developed to produce a viral titer up to 10⁸ infectious viral particles per milliliter without concentration of the cell culture supernatant, preclinical or clinical experiments that require very large amounts of vectors in a minimal volume remain difficult to perform.⁴² Strategies using MLV-derived vectors will benefit from the development of new production strategies. For example, the env protein, which controls the interaction of the viral particle and the receptor at the cell surface of the target, can be replaced with env protein derived from other viruses such as the g protein of the Vesicular Stomatitis Virus, which allows retroviral particles to be concentrated by ultracentrifugation.⁴⁴⁻⁴⁶ In addition, MLV-derived retroviral vectors transduce only cells undergoing at least one mitosis step. Retroviral vectors are thus efficient to transduce HCC cell lines *in vitro* that actively proliferate, but remain poorly efficient to transfer a transgene of interest into HCC cells *in vivo* that moderately proliferate.⁴⁷ As developed in every vector system, tissue-specific expression is a major challenge when designing retroviral vector backbones.⁴⁸

Lentivirus-derived vectors: a step beyond? Since 1996, increasing evidences support the interest in using lentivirus-derived vectors either derived from Human Immunodeficiency virus (HIV) type-1, the most widely used lentiviral vector, Simian Immunodeficiency Virus (SIV), Feline Immunodeficiency Virus (FIV) or Equine

Infectious Anaemia Virus (EIAV). Lentiviral vectors are retroviral vectors, which, in some instances, have been shown to be able to transduce low- or nonproliferating cells such as neurons, nonstimulated normal hepatocytes or growth-arrested HCC cells conversely to MLV-derived vectors.^{49–55}

MLV-derived vectors and HIV-1-derived vectors share several features. The gag, pol and env coding sequences, along with the rev, vpr, nef, vif, vpu and tat coding sequences which encode regulatory and accessory proteins, have been eliminated from the vector backbone. Considering that the promoter located in the LTR get silenced in transduced cells in the absence of production of the transcription factor Tat, the transgene is placed under the control of an exogenous promoter. *Trans*-complementation with HIV-1-derived gag, pol and rev proteins enables the production of infectious viral particles assuming the presence of the Rev Response Element (RRE) in the vector backbone. The majority of the experiments reported take advantage of self-inactivating vectors that promote the elimination of the viral promoter elements in the transduced cells. Most of the experiments have been carried out with VSV-g proteins to pseudotype the viral particles.^{49,51–54} Recent reports depict the use of alternate envelopes such as the env Ross River Virus or sendai virus f protein to improve gene transfer into hepatocytes.⁵⁰ Safety concerns regarding the emergence and the detection of RCV slowed down the starting of clinical trials with this vector. It is to be noted that no HIV-like RCV arising from the production of defective lentiviral vectors has been reported yet.

We recently showed that HIV-1 lentiviral vectors efficiently transduce HCC cells *in vitro* compared to MLV-derived vectors or adenoviral vectors. Transduction of nonstimulated normal hepatocytes with HIV-1 lentiviral vectors remains controversial. The reported discrepancies could originate from the origin of the cells and the presence of stimulating proteins in the culture medium, and the maintenance or the absence of an HIV-derived *cis*-active element called the DNA Flap sequence, which could favor or diminish, respectively, the ability of the lentiviral vector to integrate the transgene in the target cell genome.^{56–58} The low transduction of normal hepatocytes with lentiviral vectors, which do not contain the DNA Flap sequence, could be considered as a safety strategy to avoid the transduction of normal hepatocytes. Indeed, experiments performed in the laboratory with intra-arterial injection of DNA Flap-less HIV-1 vectors in the liver of rat exhibiting DEN-induced HCC clearly showed an efficient transduction of HCC cells with the absence of transduced normal hepatocytes as assessed using the LacZ marker gene activity (R Gérolami *et al*, unpublished results). This property cannot be used as a key safety alone since it should be considered that in the context of HCC, which is characterized by the development of regeneration nodules and an increased proliferation index of parenchyma cells, normal hepatocytes may exhibit an increased sensitivity to lentiviral transduction.

Numerous improvements, that is, the use of tissue-specific promoters, insertion of *cis*-active elements able to

increase the transcription efficiency, etc., still have to be assayed, but lentiviral vectors can already be foreseen as very powerful gene transfer tools to be tested to transduce HCC cells or normal hepatocytes *in vivo*.

Alternate vectors to transduce HCC: alternate dreams? Vectors derived from the SV40 have been tested by replacing the large T antigen coding sequence with the transgene of interest leading to the generation of infectious particles that do not replicate in transduced cells and exhibit a low immunogenic profile. SV40-derived vectors can transduce quiescent and replicating cells and integrate the transgene in the genome of target cells. Effectiveness of SV40-derived vectors in the genetic manipulation of HCC remains to be firmly demonstrated.²⁴

Baculovirus vectors which can replicate in insect cells, have been reported to be able to transduce human hepatocytes without further replication. They have also been reported to be able to transduce HCC *in vitro* but exhibit a high sensitivity to complement, which could impair their use in humans.^{24,59,60}

Strategies aimed at improving *in situ* stable gene transfer have been approached using chimeric vectors, which combine adenoviral vectors and retroviral vectors. Adenoviral vectors are designed to transfer a retroviral vector backbone and the gag, pol and env *trans*-complementing elements and injected *in vivo*, thus leading to a local production of retroviral particles with subsequent transduction of neighboring cells. These vectors have been tested to transduce HCC cells but remain very difficult to envisage for a clinical trial due to safety concerns.⁶¹

Infection of tumor cells by herpes simplex virus 1 (HSV) results in cell death by viral oncolysis, and genetically engineered HSV-1 mutants have been generated to transduce and eliminate *in vivo* HCC cells.^{62,63} The difficulty in generating and manipulating HSV vectors in a clinical context makes difficult the present clinical development of these vectors.

Nonviral vectors

Expression plasmid and additives. Gene transfer into liver cells using DNA has to be considered a very interesting strategy for gene therapy of HCC. DNA molecules, exhibiting the coding sequence of interest and the regulatory elements involved in the transcription mechanism, are used either under circular or linear configuration and can be injected as naked molecules or combined with chemical additives or physical constraints able to increase transduction efficiency.^{64–68} Most of the DNA molecules transferred in a target cell localize in the cytoplasm and only minor integration events in the genome can be observed. DNA molecules exhibit a low immunogenic potential and it is possible to easily produce large amounts of therapeutical product under clinical grade conditions. In this strategy, systemic administration has been intensively investigated assuming the liver, as a first-pass organ, exhibits strong abilities to retain DNA

molecules; however, intraportal injection, direct injection of the gene therapy product in HCC has also been attempted.^{69,70} Considering that the low gene transfer efficiency observed with this strategy would imply the injection of very large amounts of DNA molecules to efficiently hit HCC *in vivo*, numerous efforts are focused to improve delivery of the expression unit using chemical additives such as PEI, liposomes or polyamidoamine dendrimers, etc. in synergy or not with helper additives of viral origin.^{71,72} Some compounds such as asialoglycoproteins combined with DNA molecules can specifically improve the transduction of hepatocytes.^{24,73} The targeting of the tumor cell can be obtained with the coupling of the binding peptide directed to membrane tumor-associated antigen with DNA molecules.^{74,75}

The Antisense approach. The expression of genes can be affected by the transfection of noncoding oligonucleotide molecules complementary to sequences localized in the selected target gene. The transcription of the target gene can be inhibited following the formation of a triple-helix structure between the double-strand DNA and the oligonucleotide. This strategy can also inhibit the translation of the protein by hybridization of the oligonucleotide with the messenger RNA that masks maturation sites such as splicing sites, promotes the digestion of the chimeric molecules by endogenous RNases H or cleaves the mRNA using autocatalytic sites with the ribozyme strategy.⁷⁶

Viral vector and transgene: therapeutic or deleterious partners to be controlled

It should be noted that both the viral vector used and the transgene of interest can synergize to induce an adverse effect if the vector promotes the transduction of normal cells. For example, the concomitant transduction of normal hepatocytes and HCC with adenoviral vectors can exert strong deleterious effects as shown in the woodchuck model and the use of HSV-TK containing adenoviral vectors due to intrinsic toxic effects associated with the HSV-TK gene product.^{20,26} The potential adverse effects of the production of some cytokines such as Interleukin-2 raise the interest to strictly localize and thus minimize the production to target cells.⁷⁷ Transgene expression in nonselected cells can be monitored using either selective transduction of target cells or tissue-specific expression of the transgene.^{40,78} The possibility to efficiently target selected cells has now proven to be difficult despite numerous efforts.^{79,80} In parallel, the use of tissue-specific promoters to drive the transcription of the therapeutic transgene showed some interests although low expression of some promoters, such as the promoter of the alpha-feto protein gene in rat DEN-induced HCC, could prevent the development of efficient therapeutical effect for some transgenes.^{26,40,78,81} Numerous tissue-specific promoters can be investigated assuming the combination of the relevant *cis*-active sequences able to promote tissue-specific expression and the transgene of

interest does not exceed the insert size in the selected vector.^{51,82}

Therapeutic transgene and strategies

Numerous strategies relying on direct or indirect induction of apoptosis in tumor cells or reactivation of the immune system against tumor cells have been tested to eradicate HCC and HCC-related metastases in animal models.

Using apoptosis to program the cell death

Inactivation of tumor suppressor genes has been found to be involved in numerous cancers with inactivation of p53 in about 50% of the human tumors. It is to be noted that the involvement of the deletion or the inactivation of p53 in HCC still remains controversial considering that 80% of HCC do not exhibit p53 mutation.⁸³ Some HCC exhibit p53 alteration, such as Aflatoxin B1-induced HCC with a point mutation at codon 249 in the p53 coding sequence,⁸⁴ and it has been suggested that overexpression of some viral proteins such as HBX could inactivate p53.^{85,86}

Transfer of the wild-type form of the p53 gene has been however extensively tested in HCC regardless of their p53 status. Overexpression of p53 in HCC has been shown to induce apoptosis⁸⁷ and to diminish the growth of tumor cells.⁸⁸ Intratumoral injection of an adenoviral vector able to transfer and express the p53 gene (Adp53) in subcutaneous tumors ectopically implanted in a mouse model inhibits tumoral growth. Repeated intra-arterial injections of Adp53 were also shown to be efficient in altering the growth of orthotopically implanted tumors developed from HCC cell lines exhibiting a mutated p53 genotype.⁸⁹

Only a few experiments have been reported using more complex tumor models. Transfer of the p53 gene into multiple HCC naturally occurring in transgenic mice showed that overexpression of p53 does not exert a therapeutic effect in this model, but it is to be noted that in this study, the authors have chosen an intraportal route to inject the adenoviral vector, which favors transduction of normal hepatocytes.⁹⁰ In chemically induced HCC in the rat, reiterative intra-arterial injection of Adp53 results in a moderate therapeutical effect. Low transduction efficiency *in vivo*, or intrinsic sensitivity of HCC to p53-induced cell death, as suggested by discrepancies observed using HCC cell lines as targets, could explain some disappointing results.^{88,91} Combined variation of the expression of p53 and other proapoptotic proteins such as Rb has been suggested to be involved in the tumorigenic mechanism, thus requiring the HCC to show specific expression profiles of different proapoptotic genes to be able to respond to a therapeutic p53 overexpression.⁹² For example, conversely to HCC exhibiting only a p53 mutation such as HuH7, or HCC that do not exhibit p53- or Rb mutation, p53- and Rb double mutants have been shown to be very sensitive to wild-type p53

overexpression.⁵⁹ Altogether, these data should restrict the interest of using the overexpression of p53 alone to eradicate tumor cells. Moreover, the transduction of proapoptotic genes such as p16, p27, E2F-1 or E1A alone or in combination with p53 has already been shown to be very promising. Sandig *et al*⁵⁹ reported the simultaneous transduction of the genes coding for p53 and p16 leading to a synergistic antitumoral effect in subcutaneously implanted tumors in mice. The possibility to combine the transfer of proapoptotic genes with conventional anticancer chemo- or radiotherapy has also been envisaged as shown with the retroviral transfer of p53 in Hep3B cells, which sensitizes Hep3B cells to platinum derivatives treatment *in vitro*.⁸⁸ The treatment of chemically induced HCC by anti-Bcl2 antisense has also been attempted and showed the increase of the apoptosis in dysplastic nodules and HCC in injected animals compared to controls.⁹³ Regression of xenografted human hepatomas using the chicken anemia apoptin has been recently described.⁹⁴ Considering the vast complexity and potential of the apoptosis, it is obvious that the apoptotic-induced elimination of HCC will be frequently revisited in the future.

Although the intratumoral injection of five HCC patients with Adp53 expression unit as naked DNA was reported, no clinical result has been published yet. A partially deleted adenoviral vector that still expresses the E1A protein and induces apoptotic and necrotic mechanisms has been injected into patients without efficient clinical response.³³

The suicide strategy

The HSV-TK/GCV treatment. This strategy relies on the expression of an enzyme that promotes the catalysis of a prodrug into a toxic compound in the target cell.^{95–97} To this purpose, HSV-TK expression, which confers sensitivity to ganciclovir (GCV) has been largely experienced. GCV is phosphorylated by HSV-TK prior to be di- and triphosphorylated by endogenous enzymes in the target cell. Incorporation of this abnormal triphosphorylated nucleoside analog in the replicating DNA leads to apoptotic-mediated cell death in cells undergoing mitosis. This approach has been originally tested to treat glioma considering that, as it is the case in HCC, cells surrounding the tumor exhibit low proliferative capacity and only tumor cells should be eliminated with this strategy.^{98–102} However, it should be noted that the phosphorylated GCV exerts a toxic effect on normal hepatocytes, presumably through a mitochondrial toxicity.^{103,104} Cells expressing HSV-TK have been shown to induce the death of neighboring cells that do not express the transgene, leading to the possibility in some cases to eradicate a whole tumor mass with only 10% of transduced tumor cells.^{105,106} This so-called bystander effect requires the presence of gap junctions between neighboring cells and genetically manipulated cells, which allow the transit of phosphorylated analogs and the subsequent death of neighboring cells even if these cells do not express HSV-TK.^{107–109} Considering the difficulties to

transduce 100% of the tumor cells, the bystander effect has been shown as a key parameter for the therapeutical impact of this strategy. Moreover, in some cancer cells, it has been proposed to overcome the absence of relevant gap junctions by overexpressing selected connexins along with the HSV-TK gene.^{110–113} However, the bystander effect has also been shown to be efficient in normal cells and could emphasize the hepatic toxicity of the HSV-TK/GCV treatment when normal cells are fortuitously transduced. In some immunocompetent animal models of HCC, a distant bystander effect relying on the activation of the immune system has been described, which synergizes with the direct elimination of tumor cells.^{114–117}

HSV-TK/GCV treatment has been extensively experienced *in vitro* and in animal models either with tumors obtained following injection of HCC lines or chemically induced HCC as shown in DEN-induced HCC in the rat model.^{104,118} In this last model, using adenoviral vectors to transfer the HSV-TK gene, we have shown that a unique intratumoral injection of the viral vector with daily administration of GCV allows strong reductions of large-size tumors. For small-size tumors, intra-arterial injection in the hepatic artery has led to a significant decrease of the number of tumors in treated rats compared to control. However, the expression of the HSV-TK gene induces lethal hepatitis, thus emphasizing the interest of using promoters able to drive the expression of the transgene in the tumor cells. In this prospect, the use of the alpha-fetoprotein promoter to drive the expression of the HSV-TK gene showed moderate therapeutic efficiency compared to approaches using a strong ubiquitous promoter, which can be due either to low expression of the AFP promoter or heterogeneous expression of the alpha-fetoprotein in HCC cells.¹¹⁹ In parallel, retroviral vectors that transduce only dividing cells have also been tested to transfer the HSV-TK gene in tumor cells without affecting normal hepatocytes, but these experiments showed low transduction efficiency in HCC implanted in a nude mice model and would benefit from repeated injections of the retroviral vector to promote an efficient therapeutic impact.¹²⁰ We showed that *in vivo* delivery of a lentiviral vector expressing an HSV-TK::EGFP fusion protein resulted in a selective transduction of tumor cells. An intratumoral injection of LeTk followed by the administration of GCV resulted in the regression of small tumors and growth arrest of large tumors. No evidence for hepatitis was seen in nontumorous liver accordingly with a very low transduction of normal hepatocytes (R Uch *et al*, unpublished results).

Moving to new suicide genes keeping the bystander effect on. Several suicide strategies have been described or are under investigation.¹²¹ The *Escherichia coli* cytosine deaminase, which promotes the production of toxic 5-fluorouracil (5FU) from 5-fluorocytosine (5FC), should exert low hepato-toxicity with a stronger bystander effect.^{62,122–125} However, fulminant hepatitis have already been described following systemic injection of 5FU,¹²⁶

and the therapeutical impact of the 5FU treatment to eradicate HCC still remains controversial. Although efficient *in vitro* and in animal models, the interest of using the cytosine deaminase/5FU strategy still remains to be confirmed. The possibilities to use the rabbit cytochrome *p*450 4B1, which induces cell death following the treatment of transduced cells with 4-ipomenanol, or the use of *E. coli* purine nucleoside phosphorylase gene, which converts purine analogs into toxic metabolites, are now under investigation and show some interest as an alternative to the HSV-TK/GCV strategy considering they also exhibit a strong bystander effect.^{70,127} The comparison between the purine nucleoside phosphorylase (PNP) strategy and the HSV-TK/GCV showed the interest of using the PNP/fludarabine combination, which exhibits a stronger killing effect than HSV-TK/GCV in HCC cells line with a mutated *p*53 genotype.¹²⁸

From the immunomodulation to the generation of the antitumoral response

Viral or nonviral vector-mediated transfer of genes coding for immunomodulatory molecules or Tumor Associated Antigens, TAA, may generate or reactivate the immune response against tumor cells. Numerous strategies have thus been attempted. In addition, the local production of optimal doses of some cytokines such as Interleukin-2 or interferons could induce a more efficient therapeutical immune response than systemic administration of large doses and would prevent serious adverse effects arising from the systemic administration of these molecules.

Numerous cytokines have been approached to generate an immune response against tumor cells in animal models. Stable expression of Tumor Necrosis Factor Alpha, Interleukin-2, Interleukin-12, Interferon regulatory factor-1 or chimeric cytokines decreases the tumorigenic potential of retrovirally manipulated HCC and, in some instances, induces an immune response against control parental cells implanted in the same animal.^{77,129–134} The HSV-TK/GCV treatment has also been shown to correlate with the induction of an immune response against tumor cells called the “distant bystander effect” that induces the regression of orthotopic HCC tumors at a distant site from the reinjected tumor.¹¹⁶ Transfer of immunomodulatory cytokines such as Interleukin-12 and GM-CSF or the recruitment of accessory cells with the chemoattractant protein-1 has been proposed to potentiate the antitumor effect of the HSV-TK/GCV treatment.^{130,135,136}

Several mechanisms have been reported to explain the low immunogenic potential or the absence of immune recognition of the tumor cells including the lack of expression of the Major Histocompatibility Complex B7 molecule at the membrane of the tumor cells. Gene transfer-mediated expression of B7 molecules has shown reduction of the tumoral size of genetically manipulated cells and induction of an immune response against parental cells that do not express B7.¹³⁴

The manipulation of professional antigen-presenting cells such as dendritic cells is considered as a very

promising strategy in the treatment of numerous cancers but relies on the characterization of TAA.¹³⁷ The transduction of the alpha-fetoprotein gene in dendritic cells, which have been shown to be overexpressed in some HCC, lead to the regression of HCC implanted in the mouse model.^{138,139} As an alternative to the direct manipulation of APC, the injection of a recombinant vaccinia virus able to specifically express the murine alpha-fetoprotein along with vectors expressing immuno-modulatory molecules showed tumor regression in mice.²² It is of note that the risk of autoimmune disease has been reported using this tumor-specific epitope as a target for the immune system during liver regeneration in mice.¹⁴⁰

Promising results were obtained with tumors arising from the injection of HCC cell lines, but only few and poor results have been described with HCC models close to human pathology. Morel et al¹⁴¹ showed that genetic manipulation can induce an immune tolerance the emergence of tumors and increase the occurrence of HCC in transgenic mice overexpressing the Simian Virus Large T antigen. In the model of adenovirally transduced DEN-induced HCC in the rat, no distant bystander effect was observed.¹⁰⁴ Interestingly, the activation of an antitumoral effect mediated by T lymphocytes and natural killer cells has been obtained using adenoviral transfer of the CD40 ligand in the Buffalo rat model.²³

Inhibition of the angiogenesis

The inhibition of neovascularization, which correlates with and controls tumoral growth can drastically impair tumor development. The expression of a soluble receptor for the vascular endothelial growth factor (VEGF) as a competitor to the normal cell membrane counterpart has been shown to diminish the tumorigenicity of tumor cells in several models including breast cancer and melanoma.^{142,143} Angiogenesis inhibitors such as angiostatin or endostatin can also be used to alter the vascularization of the tumor.^{144,145} It has also been suggested that overexpression of *p*53 could inhibit tumor angiogenesis thus leading to the inhibition of tumoral growth *in vivo*.¹⁴⁶ In addition, the transfer of Interleukin-12 could induce tumor regression using the inhibition of angiogenesis along with the activation of natural killer cells, leading to the possibility controlling angiogenesis through several distinct mechanisms.¹⁴⁷ Although the strong vascularization of HCC can be foreseen as an interesting target,¹⁴⁸ no result has yet been reported using gene transfer to control angiogenesis in HCC.

Playing with the biological activation pathways

The inactivation of signal transduction pathways involved in the malignant phenotype, which induces the death of tumor cells via mechanisms related to the apoptosis or the immune reaction, shows some interest in the reduction of the tumorigenic potential of HCC and can be approached using the antisense strategy or the expression of a specific repressor able to interfere with the selected biological pathway.^{149–155}

Antisense against N-ras or against the fibroblast growth factor-2 mRNA inhibits the proliferation of HCC *in vitro* and reduces the tumorigenicity of the cells in the nude mice model.¹⁵³ The low proliferative ability and tumorigenic potential of HCC cells treated by antigens anti-IGF-1 has been demonstrated to correlate with apoptosis mechanisms and the induction of an immune response against tumor cells due to IGF-1-mediated re-expression of MHC B7 molecules at the surface of tumor cells.^{150,154} Most of the reported results depict experiments performed with HCC cells manipulated *ex vivo* to overexpress the antisense oligonucleotide inhibitor prior to be reinjected in the animal, which does not reflect the therapeutical potential of this approach.

The control of transduction signal pathways by transfer of regulatory proteins has also been proposed using the adenoviral transfer of a nuclear factor kappa B repressor gene,¹⁵⁶ or the overexpression of a negative regulator of the JAK/STAT pathway.¹⁵⁷

Conclusion

Gene therapy strategies focusing on the treatment of HCC emerged about 10 years ago with the suicide HSV-TK/GCV approach, and showed the development of numerous alternatives according to the improvement of the gene transfer tools and the development of new approaches to promote the elimination of tumor cells. However, the absence of relevant therapeutical results in human markedly stresses the interest of reassessing the proposed strategies in a more complex context such as animal models exhibiting virally or chemically induced HCC. Among the vectors envisaged lentiviral vectors are considered as important tools to be tested; however, as it is the case for any vector, it is evident that the full potential of the lentiviral vector requires numerous improvements that will arise from vector development projects synergizing the industry and academic research, and relying on both animal model experiments and phase-I clinical trial results. Numerous therapeutical genes or biological activities of interest have already been investigated and have show promises in the possibility to control the development of HCC. Whether the effectiveness of these therapeutic transgenes only relies on the transduction efficiency will have to be demonstrated. It should be strongly envisaged that the combination of transgenes of interest or the combination of gene therapy with conventional therapeutical approaches will greatly improve the treatment of HCC. Emerging fields such as the possibility to generate normal liver cells from stem cell populations also raise the possibility to genetically manipulate liver stem cells to protect them from further ablative cancer treatment.¹⁵⁸ This would allow the combined use of destructive gene transfer into cancer cells and protective genetic manipulation of normal regenerating cells that would potentially synergize their benefit for the patient. Finally, considering that most of the HCC arise from cirrhosis or from some viral

infections, prevention of these first pathologies by gene therapy should be envisaged.

Altogether, it can be envisioned that gene therapy will be considered as a powerful strategy or, in some cases, as a synergistic treatment to conventional approaches. The development of the gene transfer method for the treatment of HCC should be envisaged as a very promising effort for the patients.

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