Gene therapy imaging in patients for oncological applications

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Abstract. Thus far, traditional methods for evaluating gene transfer and expression have been shown to be of limited value in the clinical arena. Consequently there is a real need to develop new methods that could be repeatedly and safely performed in patients for such purposes. Molecular imaging techniques for gene expression monitoring have been developed and successfully used in animal models, but their sensitivity and reproducibility need to be tested and validated in human studies. In this review, we present the current status of gene therapy-based anticancer strategies and show how molecular imaging, and more specifically radionuclide-based approaches, can be used in gene therapy procedures for oncological applications in humans. The basis of gene expression imaging is described and specific uses of these non-invasive procedures for gene therapy monitoring illustrated. Molecular imaging of transgene expression in humans and evaluation of response to gene-based therapeutic procedures are considered. The advantages of molecular imaging for whole-body monitoring of transgene expression as a way to permit measurement of important parameters in both target and non-target organs are also analyzed. The relevance of this technology for evaluation of the necessary vector dose and how it can be used to improve vector design are also examined. Finally, the advantages of designing a gene therapy-based clinical trial with imaging fully integrated from the very beginning are discussed and future perspectives for the development of these applications outlined.

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Gene therapy of cancer: general introduction

Recent progress in basic science has significantly increased understanding of the mechanisms of carcinogenesis and tumor progression, as well as the immune response elicited towards the tumor. This body of knowledge, along with the unraveling of the human genome [1, 2], has expedited the development of new therapeutic strategies which may be able to circumvent resistance mechanisms towards conventional therapeutic approaches. In this environment, gene transfer has emerged as a new and promising therapy for many diseases [3–5], and hundreds of clinical trials have been designed for the treatment of inherited disorders and a wide variety of acquired diseases. Almost 66% of all gene therapy clinical trials are in fact devoted to the treatment of cancer [6, 7].

The underlying principle of gene therapy is conceptually simple: the introduction of genetic materials into cells to produce a beneficial biological effect leading either to cure/remission of the treated disease or to at least improvement of the clinical status of the patient. However, incorporation of genetic materials into target cells often cannot be easily achieved without packaging them into molecular vehicles, called gene therapy vectors [8–10]. Ideally, these vectors should be able to harbor large DNA constructs, transfect or transduce target cells efficiently, allow for controlled and targeted gene expression, be safe for both the patient and the environment, and be produced at an adequate amount and reasonable expense. Currently, the most widely used gene therapy vectors are replication incompetent viruses [6].

In contrast to successful eradication of tumors and increased survival in experimental cancer gene therapy models, the results of cancer gene therapy trials in patients

have not been encouraging. Possible reasons for this discrepancy are inefficient incorporation of the transgene into tumor cells, an inadequate level of transgene expression in the cancer cells, and a shorter than required duration of transgene expression in the cells. Moreover, to ensure patient safety, it is necessary to determine the biodistribution of transgene expression. Even in animal cancer models, repetitive analysis of whole-body transgene expression is quite cumbersome. In the clinical setting, these studies would imply unacceptable repetition of invasive procedures that would not only put the patient at risk, but also provide only very limited information since they do not allow assessment of whole-body gene expression distribution. Thus, there is an urgent need to develop sensitive and reproducible non-invasive imaging methods that could be repeatedly and safely performed in patients. In this sense, molecular imaging has emerged as a non-invasive technology for in vivo mapping of gene expression and provides promising tools for rational progress of molecular medicine and gene therapy [11–15].

Current status of gene therapy-based anticancer strategies

Replacement of tumor suppressor genes

Tumor suppressor genes hinder abnormal cell proliferation and induce apoptosis, or cell cycle arrest in malignant cells. The main representative gene of this family is *p53*, whose protein product interferes in the biochemical pathways of many genes which regulate cell growth and differentiation [16]. Initial results of *p53*-based gene therapy clinical trials have not been too successful [17], but different approaches targeting *p53* are being extensively explored. These include induction of *p53* activity in those cells bearing a wild type gene, or correction of mutant *p53* to restore wild type functions, or even the manipulation of *p53* signaling indirectly, whether through upstream regulators or through its downstream effectors.

Antisense strategies

Antisense strategies are based on blocking the activity of the altered gene at either the transcriptional or the translational level. One approach involves antigene oligonucleotides binding to DNA through Hoogsteen hydrogen bonding, forming a non-functional triple helical structure. This strategy has the advantage of a limited number of targets per cell for the therapeutic oligonucleotides [18]. A second approach affecting transcription is the use of double-stranded oligodeoxynucleotides bearing the consensus binding sequence of a specific transcription factor that are specifically recognized and bound by the target factor. Interaction with the decoy results in the inability of the protein to subsequently bind to promoter regions of target genes, resulting in a reduction or blockade of transcriptional activation [19]. Finally, single-stranded antisense oligonucleotides [20, 21] can bind to mRNA through Watson-Crick pairing, inhibiting the process of mRNA translation, or leading to the activation of the enzyme RNAse-H, which can degrade the bound mRNA. Further refinement of this strategy permits even the design of catalytic RNA oligos (i.e., ribozymes) able to destroy the targeted sequence once bound to it [22].

Drug sensitization by transduction with "suicide genes"

Gene-directed enzyme prodrug therapy [23] (which has traditionally been called "suicide gene therapy") is a two-step therapeutic approach for cancer gene therapy. In the first step, the transgene is delivered into the tumor and expressed. In the second step, a harmless prodrug is administered and selectively activated by the expressed enzyme, leading to the production of a toxic metabolite that subsequently induces cell death. The effect of this kind of therapy is to induce apoptosis not only of the transduced cells but also of the surrounding ones owing to the bystander effect [24, 25]. Through this mechanism, the toxic metabolites are transferred from the transduced cells to the neighboring ones via gap junctions or apoptotic vesicles.

The most commonly used "suicide system" is the herpes simplex virus type 1 thymidine kinase (*HSV1-tk*) gene in combination with ganciclovir; although several different systems such as cytosine deaminase/5-fluorocytosine, cytochrome p450/cyclophosphamide and others have also been used.

Genetic immunotherapy

Cancer cells can elicit an immune response, but it is not enough to eradicate tumor cells. The ability of cancer cells to escape the immune system can be due to down-regulation of antigen expression or major histocompatibility complex molecules, the secretion of different immunosuppressing factors, or the lack of the necessary co-stimulation [26].

The most commonly used strategy for genetic immunotherapy relies on the transfer of the genes of immune-stimulating molecules such as cytokines. Research has mainly focused on interleukin-12, whose production either by transduced tumor cells [27] or by in vitro genetically modified dendritic cells [28] might boost the immune response by activating, among others, cytotoxic T lymphocytes and natural killer cells. Other approaches include direct genetic vaccination by the antigen-encoding genes, which induce capture of the new antigen and migration of antigen-presenting cells to lymphoid organs to initiate the desired antitumor response [29].

Transfer of genes interfering with the biological program of tumor growth

Proliferation of cancer cells is associated with angiogenesis in order to provide cancer cells with the necessary nutrients

for active metabolism. Hence, targeting the proliferation of tumor neovasculature might be an effective anticancer therapy. Preclinical studies have clearly indicated that most angiogenesis-inhibiting factors exert cytostatic rather than cytotoxic effects, thus implying the need for long-term administration in order to obtain a prolonged therapeutic effect. Hence, a gene therapy-mediated approach to the delivery of antiangiogenic genes might have the potential to overcome the limitations of limited half-life, side-effects, and cost of the treatments. In addition, the potential for sustained and controlled expression are important advantages that could be achieved by gene therapy approaches [30].

Optical and MR imaging techniques and their current limitations in humans

Molecular imaging techniques have been used for many years in research laboratories but non-invasive molecular imaging in living subjects has been available only in the past decade. Three different technologies have been developed almost concurrently for non-invasive in vivo molecular imaging: optical imaging, magnetic resonance imaging (MRI) and radionuclide imaging [single-photon emission computed tomography (SPECT) and positron emission tomography (PET)].

Molecular imaging requires instrumentation capable of detecting very low amounts of reporter probes that are designed to accumulate in cells expressing a specific gene. Optical bioluminescence imaging is the most affordable and cost-effective alternative for real-time analysis of gene expression in small animals, and provides a minimal background noise since luciferase is not a natural constituent of mammalian organisms and the probes only emit light when they are activated by the enzyme. Unfortunately, bioluminescence-based approaches lack detailed tomographic information, although the imaging of deeper structures could be possible by fluorescence-mediated tomography [31, 32]. In any case, optical imaging cannot be used as a general technique in humans due to attenuation of low-energy photons.

The main advantage of MRI approaches is the exquisite spatial resolution that can be achieved. However, owing to the indirect nature of the enhancement produced by MR contrast agents, a much higher concentration of the injected material is necessary to produce sufficient image contrast. Consequently, mass limitations required to obtain sufficiently strong MRI signals remain a considerable limitation for the clinical application of MRI for molecular imaging of gene expression, although some interesting work has been done with superparamagnetic derivatives of transferrin to monitor expression of the transferrin receptor gene [33]. Recently, Genove et al. [34] have described the use of a ferritin-derived new reporter that is made paramagnetic as it sequesters endogenous iron from the organism. Such an approach avoids the need for an exogenous metal-complexed contrast agent, thus simplifying the overall procedure.

PET/SPECT imaging

Of the various imaging technologies available, the radionuclide-based approaches of PET and SPECT offer unique advantages for molecular imaging assay development. PET is an analytical imaging tool in which compounds labeled with positron-emitting radionuclides are used to measure biological processes with only trace amounts of the radiolabeled probes. Molecules labeled with positron-emitting radionuclides, injected intravenously in trace quantities, are retained in tissues as a result of binding to a receptor, cell entrapment due to enzyme-catalyzed conversion, or intracellular uptake through a transporter. Tomographic images of the distribution of the radioactivity within the body can then be generated and quantitatively evaluated [35, 36]. Due to administration of trace doses of PET imaging probes, their plasma extracellular and intracellular concentrations are typically orders of magnitude lower than the concentrations of the corresponding endogenous or pharmacological agents required to elicit a biological response. Several positron-emitting radionuclides, including carbon-11, fluorine-18, iodine-124 and copper-64, can be used to label chemical receptor and transporter ligands and enzyme substrates, making available many probes for PET imaging. In addition, PET provides high spatial resolution (just a few millimetres), can be used to generate tomographic images, and gives precise quantitative data. Furthermore, recent advances in small animal tomographs have made available the PET imaging of mice and rats at unsurpassed resolutions (see [37] and references therein), thus filling the gap between basic and clinical research and leading to a new experimental paradigm, as molecular imaging is providing the means to link in vitro experimental assays to in vivo studies [38].

Molecular imaging, as a non-invasive methodology for mapping of gene expression in living subjects, provides an emerging group of techniques that will undoubtedly have a great impact on how molecular medicine and gene therapy in humans are managed. PET imaging can easily be performed repeatedly in patients and can provide reliable quantitative estimation of the concentration of the radiotracer [39]. It can be used to define parameters not available by other techniques, such as transduction efficiency of the target tissue and duration and whole-body distribution of transgene expression. This information is critical for proper understanding of the overall gene therapy process and its clinical application [40, 41].

Reporter gene/reporter probe approach

Reporter genes are nucleic acid sequences encoding for easily assayed proteins. They are very efficient tools for monitoring the efficacy of gene delivery vehicles and of gene expression. Reporter genes can also “report” on different properties and events such as the strength of promoters, the intracellular fate of a gene product (a result of protein trafficking), and the efficiency of translation initiation signals.

By linking the signaling gene (reporter gene) to the selected promoter construct, it is possible to monitor the expression of the reporter gene using a suitable reporter probe by different methodologies (colorimetric, luminometric, radioisotopic). The most widely used reporter genes have been chloramphenicol acetyltransferase, firefly and *Renilla* luciferase, β-galactosidase, and the green fluorescent protein. However all of these systems have important limitations, as their use is restricted to cell extracts or tissue samples, thus making impossible the sequential analysis of the studied event in the same subject under different conditions and its application to gene therapy studies in humans.

The possibility of obtaining images of the sites of expression of the reporter gene (Fig. 1) would undoubtedly be very useful for different kinds of research studies. The imaging reporter gene (IRG) should ideally not be expressed in the host—or at least its expression should be confined to defined tissues or organs—while the gene product should be expressed in sufficient amount and adequately processed in the host cell to produce a protein that should ideally be non-toxic to host cells, not immunogenic, and able to metabolize or bind a complementary reporter probe [42, 43]. The radiolabeled reporter probe should only accumulate in target tissues, being trapped in an effective and stable way only in transduced cells. Non-specific binding of the probe should be as low as possible and its accumulation in the target tissue should proportionally reflect the magnitude of the expression of the reporter gene.

The reporter gene must be introduced in the target cells by any of the currently available vectors. Once in the target cells, the DNA will be transcribed to the corresponding mRNA, adequately processed, and translated into the subsequent reporter protein. After administration of the reporter probe to the subject, it will accumulate only in the target cells by any kind of metabolic trapping mediated by the reporter protein.

Several combinations of reporter genes have been used so far, but three broad categories could be considered:

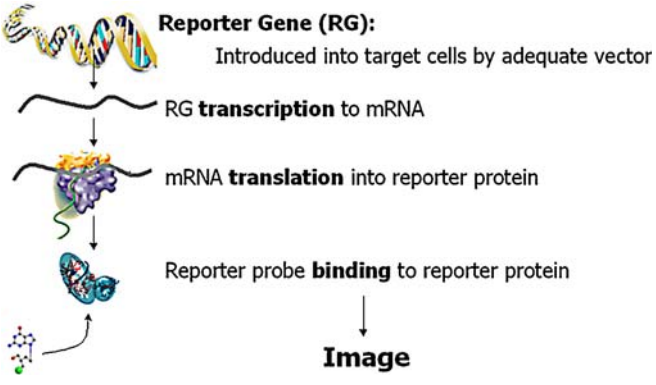

Fig. 1. The imaging reporter gene–imaging reporter probe approach. The IRG is transcribed into the corresponding mRNA, adequately processed, and translated into the corresponding protein that can in some way either metabolize or bind a specific imaging probe, thus achieving trapping of the tracer sufficient to generate an image

enzymes, receptors, and transporters. One of the earliest approaches investigated cytosine deaminase as a reporter gene with 5-[^{3}H]fluorocytosine as the reporter probe [44], but lack of sufficient accumulation of the probe due to efflux of 5-fluorouracil limits its use as an IRG. *HSV1-tk* is the most widely used nuclear IRG and a model for the enzyme-based imaging reporter transgenes (Fig. 2a).

The *HSV1-tk* gene is being used in many suicide gene therapy clinical trials. The suicide gene is usually transferred into the malignant tumour cells by infection of the target cells with recombinant viruses. Following expression of the transgene in the tumour, the prodrug (usually an acycloguanosine such as ganciclovir) is administered systemically and trapped intracellularly after its mono-phosphorylation by the HSV1-TK, an enzyme with a relaxed substrate specificity as compared with the mammalian thymidine kinases. Subsequent phosphorylation by several cellular enzymes leads to ganciclovir triphosphate, which acts as a chain terminator when it is incorporated into DNA, leading to the death of the tumoral cell expressing the transgene and also the surrounding ones by a bystander effect induced by cellular mediators.

In addition, *HSV1-tk* can be used not only as a therapeutic gene, but also as an IRG when used with an appropriate reporter probe. Two major classes of these compounds have been investigated: uracil nucleoside derivatives and acycloguanosine derivatives. Specific accumulation of some of these substrates has been demonstrated in *HSV1-tk* (or mutant *HSV1-sr39tk*) expressing cells [45–59].

An IRG can also encode for a receptor (Fig. 2b) such as the human dopamine D_2 receptor (D_2R) or the human somatostatin receptor subtype 2 (SSTR2). Both types of human receptor have limited expression in the body (the D_2R in the nigrostriatal system of the brain, and the SSTR2 mainly in pituitary gland, many carcinoid tumors, and some other types of neuroendocrine tumor). Positron emitter-labeled probes such as 3-(2-[^{18}F]fluoroethyl)spiperone ([^{18}F]FESP) and *O*-[^{11}C]raclopride are available for clinical imaging of the dopaminergic system with PET, and it has been demonstrated that ectopically expressed *D_2R* could be imaged using [^{18}F]FESP as a probe [60]. A mutant *D_2R* that uncouples ligand binding from downstream signal transduction has also been used [61]. The SSTR2 binds several naturally occurring peptides and different somatostatin analogues (such as octreotide, which is used in clinical nuclear medicine labeled with ^{111}In). Regarding positron emitter-labeled octreotide derivatives, preliminary studies have been done with ^{64}Cu- or ^{68}Ga-labeled [62, 63] compounds, although detailed studies have been carried out using *SSTR2* as a reporter gene for SPECT studies with ^{99m}Tc [64]. The feasibility of labeling octreotide derivatives with ^{18}F is currently being investigated and some preclinical evaluation of ^{18}F-labeled carbohydrated octreotide analogs has already been carried out [65].

A gene encoding a transporter protein that specifically incorporates a labeled tracer into the cell can also be used as IRG (Fig. 2c). The sodium iodide symporter (*NIS*) is probably the best example of such a gene [66], although some others, such as the norepinephrine transporter, have

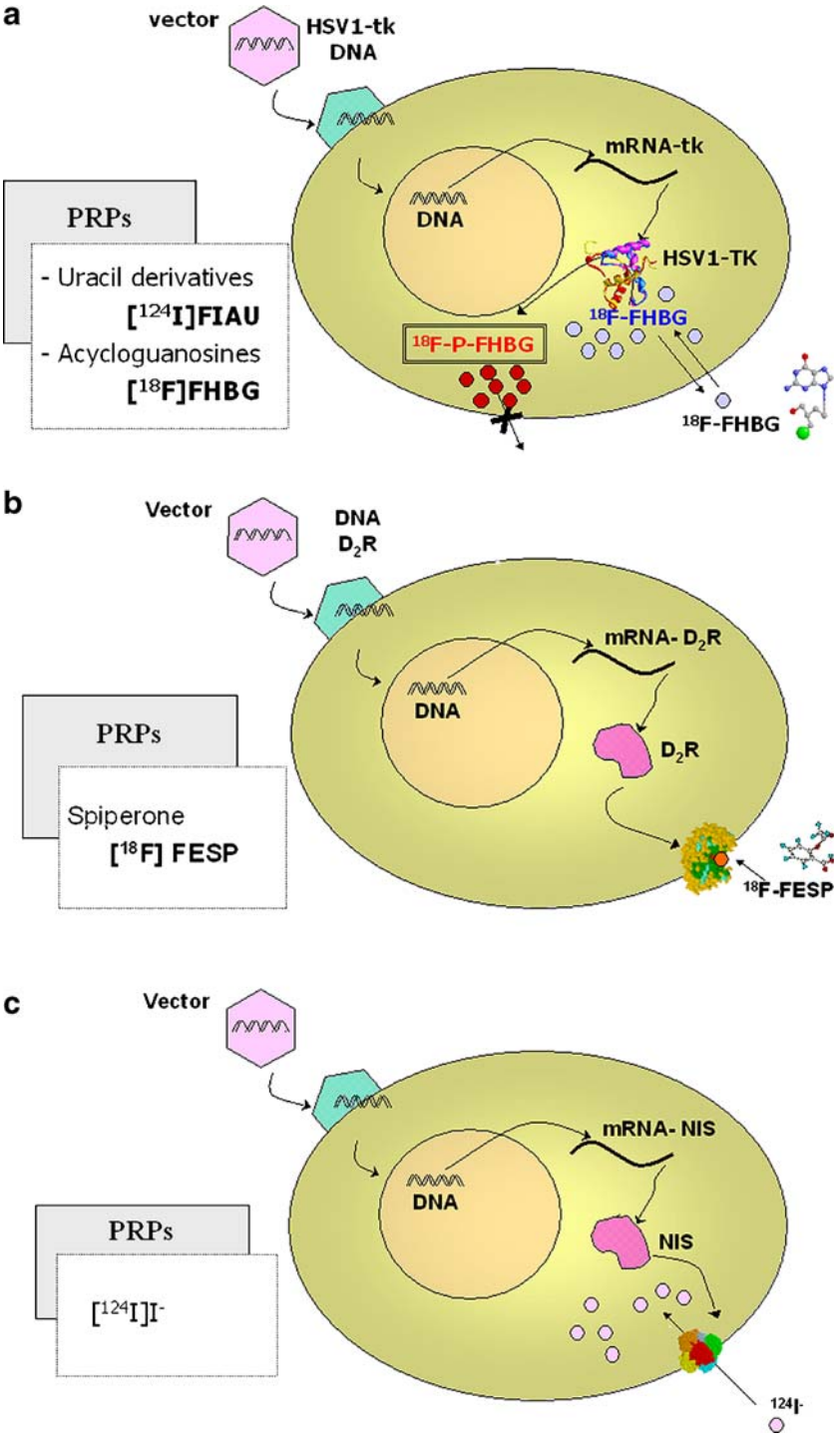

Fig. 2. The three main types of PET reporter gene: **a** enzymes, **b** receptors and **c**) transporters. Examples for *HSV1-tk*, *D_2R* and *NIS* reporter genes are shown

also been used [67]. The *NIS* is mainly expressed in the thyroid, although lower levels of expression are also present in salivary glands, stomach, thymus, breast, and other tissues. Cells ectopically expressing the NIS protein do not organify iodide as follicular thyroid cells do, but a sufficient amount of radioactivity can accumulate in transduced cells as to be visualized either by gamma camera if ^{123}I or ^{131}I is used or even by PET, if ^{124}I is used. For a review of use of the *NIS* gene as a reporter gene, see reference [68].

Every IRG–reporter probe combination has its advantages and drawbacks. Although *HSV1-tk* has greater potential to elicit an immune reaction than *hD_2R*, *hSSTR2*, or *hNIS*, the expression of a transporter or a receptor on the surface of a cell implies a complex processes of protein trafficking and membrane integration. Any system leading to signal amplification will more likely produce a sufficiently strong signal to be detected in vivo. Hence, an enzyme-based approach should ideally produce a higher signal than a receptor-based one, for the simple reason that

an enzyme molecule can catalyze the trapping of many radiolabeled molecules. On the other hand, when an intracellular enzyme is considered, the transport of the radiolabeled probe into the cell should be taken into account as well, whereas this is not a concern when extracellular receptors are considered.

If all the previous data are considered we could say that the sensitivity of both *HSV1-tk* and D_2R systems would be quite similar. In fact, a direct correlation has been found between the images of both genes administered simultaneously to the same animal [69]. Anyhow, every single case must be carefully analyzed, as specific characteristics of the target cells will probably indicate the best choice. The patterns of expression of different genes (for example those involved in the transport of nucleosides for *HSV1-tk*-based protocols) would be of capital importance for the selection; hence, a profound knowledge of the target cells is mandatory.

Molecular imaging probes for *HSV1-tk* expression monitoring

Several different positron-labeled tracers for HSV1-TK have been designed and tested both in cell culture and in laboratory animals [45, 46, 48, 50, 51, 53, 55–59], and two of them have been used in studies in healthy volunteers [70, 71] and cancer patients [72, 73]: the ^{18}F-labeled penciclovir derivative 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)-guanine ([^{18}F]FHBG) and the ^{124}I-labeled uracil derivative 5-[^{124}I]iodo-2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodouracil ([^{124}I]FIAU). The restricted availability of ^{124}I as well as its longer half-life of almost 4 days might impede serially repeated studies and could also increase radiation exposure, and the different physical decay properties of ^{124}I compared with ^{18}F would probably make [^{18}F]FHBG the most likely candidate for a widely used PET reporter probe for gene expression imaging in humans. *HSV1-sr39tk*, a mutant gene with higher sensitivity for gene expression studies with PET and higher affinity for [^{18}F]FHBG than for [^{124}I]FIAU, has also been described and successfully used for gene expression imaging in vivo [74]. Furthermore, previous studies in cell culture and in laboratory animals have demonstrated that the in vivo [^{18}F]FHBG PET signal closely correlates with transgene expression as determined by mRNA abundance and protein levels [75]. Regarding the kinetics of probe interaction with HSV1-TK enzyme, the K_M value for FHBG (K_M 0.94 μ*M*; k_{cat} 0.04 s^{-1}) has recently been published [76]. The reported K_M of thymidine for HSV1-TK is in the range 0.2–8.5 μ*M*, whereas the plasma thymidine concentration is 0.05–0.5 μ*M*. Taking into account that under physiological conditions [^{18}F]FHBG concentration is much lower than K_M, the rate constant for the interaction of the labeled tracer with the enzyme (k_{cat}/K_M) can be used as a measure of catalytic efficiency, and hence compare an enzyme's preference for different substrates. Under these conditions, thymidine is a formidable competitor for HSV1-TK sites in vivo. For high signal-to-noise ratios in the PET images, an imaging probe with high affinity and catalytic efficiency is required. When compared with other similar compounds, FHBG has the highest k_{cat}/K_M known for acycloguanosines and hence [^{18}F]FHBG can be considered the most adequate and efficient probe for in vivo imaging of *HSV1-tk* gene expression.

Coupling of target and reporter gene expression

Nowadays, one can virtually make any gene construct under the control of any promoter/enhancer elements. Promoters are *cis*-acting DNA sequences usually located upstream of the coding sequence—and relatively close to it—that are required for transcription initiation. Enhancers are short *cis*-acting DNA sequences that can enormously increase the effectiveness of promoters and can be located upstream, downstream, or even in the midst of a transcribed gene. Multiple enhancers can act cooperatively or synergistically to produce increased expression of the downstream gene. The DNA sequences of *cis*-acting elements are the binding sites for transcription factors. These proteins are called *trans*-acting elements because they can be encoded in a DNA molecule other than that containing the gene they affect. Binding of transcription factors to their cognate DNA sequence enables RNA polymerase not only to locate the proper initiation site but also to proceed with transcription efficiently.

Cis-acting elements can be either constitutive or inducible. Constitutive promoters are usually strong promoters derived from viral sequences leading to continuous transcription of the coding sequence regardless of the target cell. Inducible promoters can be switched on either in a tissue-specific manner or by a wide variety of chemical mediators or even physical stimuli such as heat or radiation (and consequently can also be turned off when the cells are deprived of the activating stimulus). Inducible promoters usually rely on the presence of the appropriate transcription factor only in some cell types, or on the chemical activation of an otherwise inactive form of the transcription factors by a chemical mediator.

The ideal way to monitor the expression of a therapeutic gene (TG) is to use a specific imaging probe that can directly detect its expression (as is the case for *HSV1-tk*). However, for most TGs a direct imaging probe is not available. The development of a new probe and its in vivo validation for each single TG would be a time-consuming and costly effort and for many genes the design of an imaging paradigm would not even be possible. Consequently, the most reasonable alternative would be to use a handful of well-characterized reporter genes for indirect imaging of TG expression. These experimental approaches would necessarily require the use of more complex systems, such us fusion gene constructs, bidirectional promoter vectors, bicistronic vectors, dual promoter construct, or simply co-vector administration (Fig. 3).

The most straightforward approach is co-administration of two gene delivery vectors, which are identical in every way except that one carries the reporter and the other the

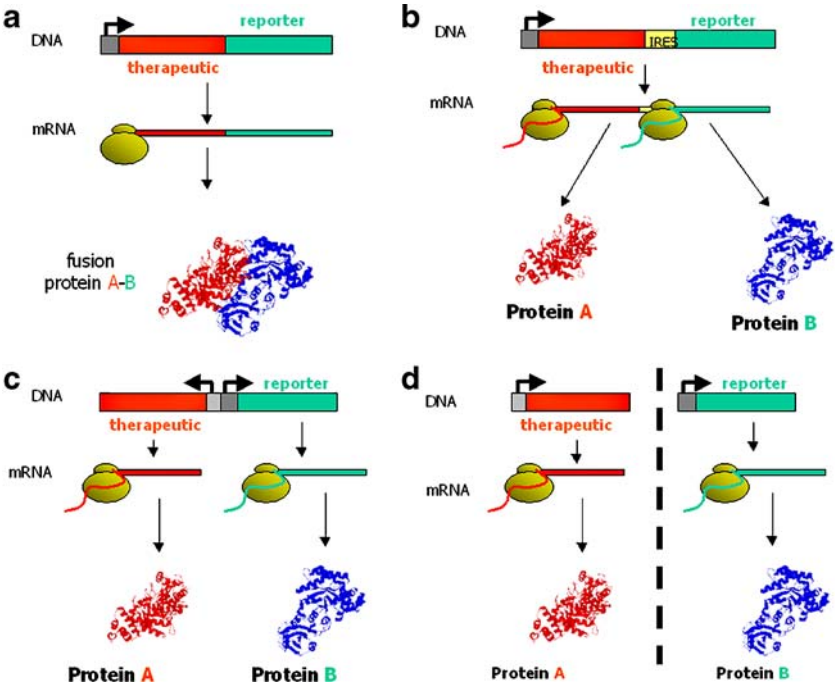

Fig. 3. Coupling target and reporter genes. The fusion protein approach (**a**) produces from a monocistronic transcription unit a hybrid protein that retains the activities of both the reporter and the TG. The use of bicistronic transcription units with *cis*-linked genes joined by an IRES (**b**) permits the synthesis of two different polypeptides from a single mRNA. The use of bidirectional promoters (**c**) produces two different mRNA chains (and hence two proteins) from a single DNA molecule. Another possibility is the use of two identical vectors (**d**), except that one carries the reporter and the other one, the TG

TG [77]. Since both vectors are identical and the expression of both the IRG and the TG is regulated by an identical promoter, when they are co-administered in equal amounts it is possible to achieve correlated expression of the IRG and the TG at the target tissue. The greatest advantage of the co-vector administration approach is that a single IRG vector can be used to track the expression of many different TGs, without the need to produce a complicated TG/IRG construct. While any single cell would not be infected with equal amounts of the two vectors, in the macroscopic scale of most clinical imaging modalities this would not pose a significant problem. However, when the double vector approach is used, the viral burden has to be increased substantially, increasing the chance of immunological reactions. Furthermore, additional mechanisms of transcription regulation could affect correlation between the expression of both genes, as some kind of either positive or negative interaction could be produced.

The use of fusion gene technology results in a fusion protein, a unique polypeptide that retains the biological activities of the two different proteins. Imaging the reporter protein part of the fusion polypeptide would give direct information on the other component of the hybrid. Although this approach using a fusion IRG–TG has already been used [78–80], the practical problems of making fusion constructs where both the therapeutic and the imaging component remain functional are likely to be complex to surmount. Every single case would have to be studied carefully, as the fusion protein could lead to a non-functional protein due either to folding problems or to intracellular localization of the final product, or even just to a decrease in the activity of any of the components of the chimeric protein that would impede its clinical use. However, interesting advances have permitted the design of fully functional, even triple-modality reporter genes for multimodality imaging [81].

The use of bicistronic transcription units with *cis*-linked genes joined by an internal ribosomal entry site (IRES) is another experimental approach. The IRES enables translation initiation within the bicistronic mRNA, thus permitting the synthesis of two different proteins from a single mRNA chain. However, both cistrons can be translated at different rates; usually the translation of the first cistron upstream of the IRES is severalfold greater than the IRES-mediated translation of the second downstream cistron. Although somewhat ambiguous results could be obtained owing to the differential expression rate of the two genes, this strategy would permit a single imaging gene to be used with ideally any TG. Several studies have reported successful imaging of IRES constructs [82, 83] and demonstrated that proportional co-expression can be achieved [84]. The aforementioned attenuation of the transcriptional unit placed distal to the IRES compared with the proximal one would not pose a serious problem if it were constant. But it seems to vary from one tissue to another, complicating substantially the interpretation of the images in terms of quantification of the TG expression. However, recent advances in the development of the so-called superIRES (SIRES) have demonstrated that SIRES-based bicistronic vectors significantly augment the levels of expression of the downstream gene [39].

Bidirectional vectors, in which the two genes are expressed from a common promoter, are another alternative. Some work has been done in this field with encouraging results, a good correlation being found between the expression of the two genes [85, 86]. In summary, it seems

that there is not a single universal solution for coupling therapeutic and reporter genes. Each case has to be analyzed carefully, and the most adequate experimental approach selected.

Uses of radionuclide imaging in gene therapy in humans

The use of gene therapy for the treatment of human pathologies has been increasing, although sometimes with somewhat discouraging results. However, many crucial questions regarding the mechanism of gene therapy remain unanswered and not much is known about the following important issues:

- Has the vector used for gene transfer been efficiently delivered to the target organ/tissue?
- Is the distribution in the target optimal?
- Has the vector reached any other organ?
- What is the expression level of the TG in different tissues?
- Did the gene transfer take place efficiently?
- Is the gene being expressed?
- Is the level of gene expression sufficient for the intended therapeutic effect?
- How long does gene expression persist both in target and in non-target tissues?
- What is the optimal time point for prodrug administration in "suicide" gene therapy protocols?
- Has the gene therapy protocol been efficient for disease cure/remission?

In this scenario, imaging of living subjects could play a pivotal role in both preclinical and clinical research in gene therapy, as a non-invasive clinically applicable method that could help to answer all these questions would be of enormous value. It would not only make possible the monitoring and evaluation of gene therapy in human subjects by defining the location, magnitude, and variation over time of gene expression, but also lead to a better understanding of vector biology and pharmacology devoted to the development of improved and safer vectors. The research both in laboratory animals and in clinical trials could also be considerably accelerated by the use of these technologies.

Non-invasive molecular imaging technologies in gene therapy can be used in several different ways:

a) To avoid invasive procedures for gene therapy monitoring
b) To evaluate treatment by imaging the functional effects of gene therapy at the biochemical level, and to determine prognosis
c) To monitor gene expression both locally and for the whole body
d) To determine the appropriate dose of the vector
e) To help improve vector design

Imaging as a non-invasive procedure for gene therapy monitoring

Until now, biopsies offer the only feasible way to know whether the vector has reached the target organ and/or whether the transgene is being expressed. However, to determine therapeutic efficacy, several biopsy samples have to be taken for each evaluation point. These procedures not only have an inherent morbidity/mortality, but also provide limited results. The biopsy procedure itself can alter the underlying tissue. Furthermore, minute tissue samples are usually taken and tissue heterogeneity may lead to a false idea of transgene expression in the target lesion. In addition, it is difficult to give accurate quantitative results of the level of transgene expression in the sample. Invasive procedures are not likely to be used repeatedly owing to the clinical complications they can produce. Finally, it is not feasible to take biopsy samples of every single organ of interest. On the other hand, PET can be used to obtain quantitative whole-body images of molecular events, non-invasively and repetitively, from the patient and can be the best method for monitoring TG expression, as will be discussed later.

Evaluation of response to gene therapy

Radionuclide imaging in oncology was originally applied for tumor detection, but its application has been broadened to monitoring the efficacy of therapy. In fact, ^{18}F-fluorodeoxyglucose (FDG) PET has already been used in cancer patients to evaluate response to radio- or chemotherapy [87–93], and in many cases FDG PET has been found to be superior to computed tomography (CT) and magnetic resonance imaging (MRI) in predicting response to therapy. Metabolic-based molecular imaging procedures such as PET and other radionuclide imaging modalities are increasingly gaining acceptance over anatomical-based imaging such as CT or MRI as the most appropriate procedures for evaluating response to therapy. This shift in the internationally accepted standards for response evaluation may take some time, but will hopefully permit a more accurate and realistic picture of gene therapy.

However, one of the major limitations for the routine application of FDG PET imaging for therapy monitoring is that no generally accepted cutoff values have been established to differentiate optimally between responders and non-responders. Prospective studies with a sufficient number of cases are needed to define specific parameters to differentiate between responders and non-responders for different tumors and treatment regimens.

Regarding gene therapy of cancer, PET has been used in experimental models to evaluate response to *HSV1-tk*-mediated suicide gene therapy [94–96]. Tumor perfusion, measured in ganciclovir (GCV)-treated *HSVtk*-expressing KBALB tumors after intravenous administration of ^{99m}Tc-hexamethylpropylene amine oxime, increased by 206% at day 2 after the onset of treatment [97]. In the same animals, the accumulation of the hypoxia tracer [^{3}H]misonidazole

decreased to 34% at day 3, indicating that the tumor tissue had become less hypoxic during GCV treatment. Dynamic PET measurements of [^{18}F]FDG uptake were performed in animals 2 days and 4 days after the onset of therapy with 100 mg GCV/kg body weight as well as after administration of sodium chloride. Furthermore, the thymidine incorporation into the tumor DNA was determined after i.v. administration of [^{3}H]thymidine. An uncoupling of FDG transport and phosphorylation was found with enhanced k_1 and k_2 values and a normal k_3 value after 2 days of GCV treatment [98]. The increase in FDG transport normalized after 4 days, whereas the phosphorylation rate increased. The thymidine incorporation into the DNA of the tumors declined to 25% of the controls after 4 days of GCV treatment. As the underlying mechanism, a redistribution of the glucose transport protein from intracellular pools to the plasma membrane may be considered and is observed in cell culture studies as a general reaction to cellular stress [99].

Since intervention with angiogenesis should result in functional consequences with respect to blood supply, monitoring of antiangiogenic approaches with functional imaging and histomorphometric analyses are desirable to deliver information about the biochemical and physiological effects of this treatment modality. PET with ^{15}O-labeled water has been used in a tumor model after transfer of an antiangiogenic gene, the soluble receptor for the vascular endothelial growth factor (*sFLT*). Transfer of the *sFLT* gene in this tumor model inhibited tumor growth and perfusion and induced changes in the expression of multiple genes related to the extracellular matrix, signal transduction, apoptosis, and metabolism. Since these changes in the gene expression pattern were observed mainly in tumor tissue and not in vitro, they represent reactions of the tumor to its microenvironment. Furthermore, inhibition of endothelial cell proliferation in vitro was much stronger than the inhibition of tumor growth and perfusion in vivo. This suggests that at least some of the changes are part of tumor defense mechanisms for survival in a less permissive microenvironment [100].

When restoration of a lost function or expression of a novel gene is the goal of the gene therapy procedure, it has been demonstrated that PET can be used to specifically evaluate the response to the treatment in an experimental gene therapy design of hemi-parkinsonian MPTP-treated monkeys [101].

Jacobs et al. [73] have shown that at least in one patient a glioblastoma lesion treated with a liposomal-derived *HSV1-tk* vector showed specific accumulation of [^{124}I] FIAU in a PET study performed 72 h after injection of the tracer, and that this result could in some ways be considered as predictive of the response to therapy. However, in this report it was not possible to image specific accumulation of the tracer in the treated lesions in any of four additional patients, and issues related to the blood-brain barrier have been raised.

Furthermore, very early gene therapy response evaluation or prediction is of great interest. We have recently shown that transgene expression monitoring by [^{18}F] FHBG could be used to predict the response to the gene therapy procedure [72] in cancer patients (Table 1). In this case, only in those patients in whom we observed a positive accumulation of the PET reporter probe in the treated nodule, when evaluated 30 days after the gene therapy procedure, could the clinical status be considered as stable disease (according to "anatomical" WHO criteria [102]). In contrast, those patients with [^{18}F]FHBG PET-negative studies had progression of the disease. Taking into account that the [^{18}F]FHBG PET study for evaluation of transgene expression was performed just 2 days after injection of the vector (and before starting the GCV treatment), we could demonstrate that PET can in fact be used as a valuable tool for very early prediction of response to the gene therapy procedure.

Our observation in humans is further supported by results in laboratory animals showing that only tumors accumulating [^{18}F]FHBG regress after GCV treatment [96]. In addition, these authors showed that since [^{18}F]FHBG (%ID) declines as GCV-exposed tumors regress, [^{18}F]FHBG PET can be used to monitor the effectiveness of GCV treatment. Unnecessary prodrug treatments could hence be avoided and patients could be switched to other treatment regimens at a very early stage. Furthermore, when using prodrugs, response could be serially monitored, and the dosage pattern or the duration of treatment modified when necessary in order to better achieve the desired therapeutic effect.

Very little information is available on how the gene therapy procedure can alter the uptake of radiopharmaceuticals such as [^{18}F]FDG, and this is something that has to be carefully considered. Metabolic changes induced in the transduced cells by vector delivery could modify the radiopharmaceutical uptake pattern in the lesion, thus giving either false positive or false negative results. Rigorous methodological PET protocols have to be followed, different specific radiopharmaceuticals used whenever possible, and the results evaluated taking into account the aforementioned

Table 1. Data demonstrating the use of PET imaging of transgene expression in humans as a very early predictor of response to gene therapy

Patient no.	Clinical response	Positive [^{18} F]FHBG accumulation
1	PD	No
2	PD	No
3	PD	No
4	SD	Yes
5	SD	Yes
6	SD	Yes
7	SD	Yes

Clinical response was evaluated according to WHO criteria[102] 30 days after a gene therapy-based *HSV1-tk* treatment as described in the text. [^{18} F]FHBG PET studies were performed just 2 days after injection of the vector. All the patients who showed stabilization of the disease showed a positive [^{18} F]FHBG PET signal in the treated tumor nodule

PD progressive disease, *SD* stable disease

considerations. In any case, other non-invasive diagnosis technologies have their intrinsic limitations, and considering the overall process, PET might be superior to any other individual technique. Nonetheless, gene therapy response and follow-up should ideally be done by combining several imaging modalities, along with detailed biochemical and genetic analysis, as the best way to gather as much information as possible.

Non-invasive monitoring of transgene expression in humans

PET has been used for imaging transgene expression in animals [103–105], showing the feasibility of non-invasive quantitative evaluation of gene expression by the combination of the appropriate PET reporter gene PET reporter probe. To date many rodent studies with the FHBG tracer have been performed (for a review see ref. [106] and references therein). These have included transgenic models in which the liver expresses *HSV1-tk* driven by the albumin promoter [107] and studies of adenoviral- [108] and lentiviral-mediated gene delivery [109]. In addition, a detailed tracer kinetic model that describes the pharmacokinetics of FHBG has recently been published [110]. Taken together, all of these studies show the feasibility of imaging *HSV1-tk* and *HSV1-sr39tk* (a mutant) gene expression in living subjects with [^{18}F]FHBG and PET.

Jacobs et al. [73] showed an increase in the accumulation rate of [^{124}I]FIAU in a single patient after intratumoral infusion of a vector encoding *HSV1-tk*. Peñuelas et al. [72] have recently shown, in larger series of patients, *HSV-1-tk* expression in tumoral hepatocarcinoma nodules treated by suicide gene therapy (Fig. 4). To further support this conclusion, in a particular patient in whom the vector was directly injected into the tumoral nodule in two different sites, [^{18}F]FHBG PET-CT images clearly showed two hot spots denoting specific accumulation in the treated zones (Fig. 5).

We have also observed that the signal to background ratio between the lesion and the non-treated liver increases with time after injection of the FHBG tracer (Fig. 6a). During the time of the study, the progressive accumulation of the tracer in the transduced cells together with the metabolic elimination of the tracer to bile by the non-transduced liver parenchyma increases the specificity of the signal, making the transduced lesion more clearly distinguishable a few hours after tracer administration. In some cases, early images are not able to show transgene expression whereas it is clearly demonstrated on delayed ones (Fig. 6b).

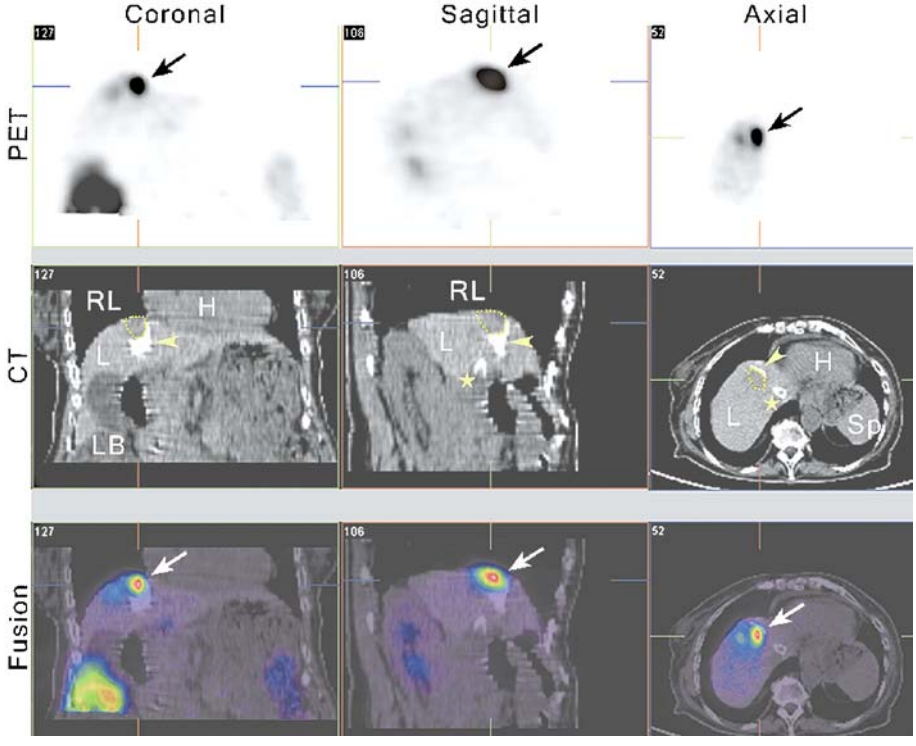

Fig. 4. PET-CT imaging of *HSV1-tk* transgene expression in humans. *From left to right*, the columns show 5-mm-thick coronal, sagittal, and transaxial slices respectively, from a [^{18}F]FHBG PET-CT study. All sections are centered on the treated tumor lesion (*yellow dotted lines* in the CT images) and show [^{18}F]FHBG accumulation at the tumor site (*arrows*). Anatomometabolic correlation can be obtained by fused PET-CT imaging. The white spots on the liver seen on the CT images correspond to lipiodol (*arrowheads*) retention after transarterial embolization of the tumor and a transjugular intrahepatic portosystemic shunt (★). Tracer signal can be seen in the treated lesion (*arrows*), while no specific accumulation of the tracer can be seen in necrotic, lipiodol-retaining regions around it. *H* heart, *L* liver, *LB* large bowel, *RL* right lung, *Sp* spleen

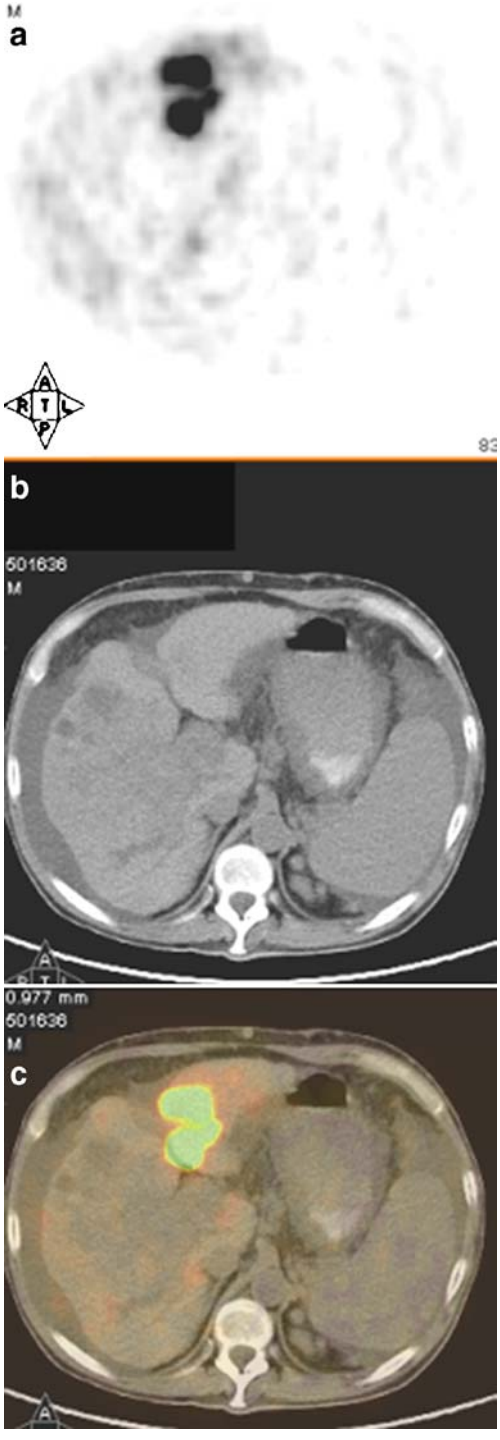

Fig. 5. Specific [^{18}F]FHBG accumulation shows *HSV1-tk* expression sites in cancer patients. PET-CT images obtained 6 h after [^{18}F]FHBG administration show two hot spots corresponding to two different injection sites of a *HSV1-tk*-encoding adenoviral vector. Images were obtained 2 days after intratumoral injection of the vector

[^{18}F]FHBG seems to have several advantages over [^{124}I]FIAU. Despite the shorter half-life of ^{18}F, we have shown that it is possible to obtain positive images of *HSV1-tk* expression using this tracer and that such images can be acquired even as soon as 50–60 min after injection when a high tumor to liver ratio is achieved in patients treated with sufficiently high doses of the vector (Fig. 7). Several investigators, including us, are in the process of investigating other PET reporter probes with improved sensitivity and specificity for *HSV1-tk* or *HSV1-sr39tk*. Our most recent findings indicate that the tritiated form of 2′-fluoro-2′-deoxyarabinofuranosyl-5-ethyluracil ([^{3}H]FEAU) has a significantly greater selective uptake in *HSV1-tk*-expressing cells than do [^{3}H]penciclovir, [^{14}C]FIAU or [^{14}C]FMAU [1-(2′-fluoro-2′-deoxy-D-arabinofuranosyl)-5-methyluracil] [111]. We have synthesized ^{18}F-labeled FEAU and are in the process of investigating its sensitivity and selectivity in comparison to [^{18}F]FHBG.

When a gene therapy procedure is considered, it is critical to demonstrate not only that it may be helpful for cure/remission of the considered disease, but also that it is safe for the patient. Great concerns were raised over gene therapy at its very inception owing to the fact that genetically modified DNA sequences (or even organisms, such as replication-incompetent viruses) are being administered to humans. An unfortunate adverse event leading to the death of a patient from multiorgan failure within 4 days of treatment during a clinical safety trial in 1999 cast significant doubts on this relatively young discipline [112]. Undesired transduction of non-target organs by viral vectors and non-specific expression of the transgene in these organs could pose serious safety risks. The use of inducible or tissue-specific promoters to permit the expression of the transgene only in the desired locations is a good approach for prevention of side-effects [113]. The induction of mutations due to integration of the transduced DNA into the host's genome in non-target cells is another great concern [114, 115], and the better way to avoid it would be to obtain extremely specific vectors that are only able to transduce specific cells [116]. Local administration of the vector (e.g., intratumoral injection) would sometimes be preferable.

We must not forget that there might be a certain level of gene expression that could not be detected by PET imaging, but with the available data it seems that the technique is so sensitive (PET can detect picomolar tracer concentrations in tissue) that such values would be so low that they would not be harmful if they existed in unintended sites.

Whole-body monitoring of transgene expression in non-target sites

Until now, there has been no reliable method to ascertain the site of vector biodistribution in all tissues of the human body, as biopsies can only provide very limited information about whole-body distribution and perhaps even yield false information owing to sampling variability and lack of quantitation.

We have shown that PET imaging of adenoviral-mediated transgene expression in cancer patients can provide information showing the absence of transgene expression not only in the treated organ outside the treated tumor nodule, but also in tissues distant from the vector injection

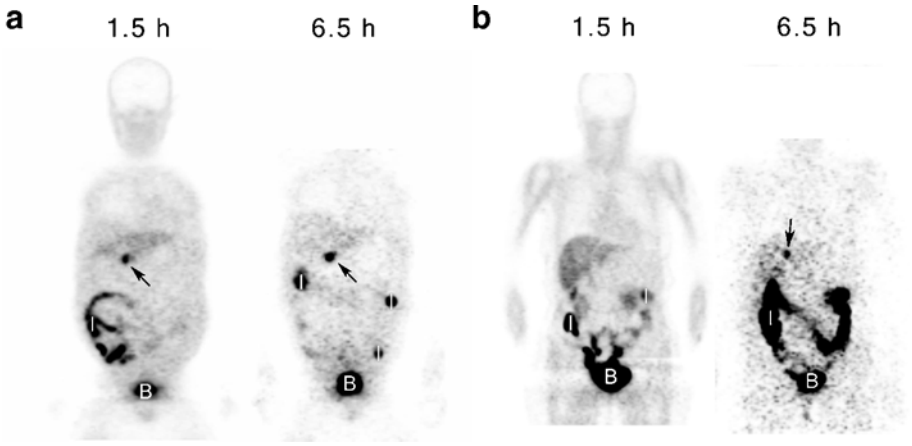

Fig. 6. [^{18}F]FHBG PET whole-body imaging can provide very valuable information showing that the expression of the transgene is circumscribed to the treated lesion and also that higher vector titers do not necessarily produce higher transgene expression. Whole-body images show that specific accumulation of the tracer (and hence *HSV1-tk* expression) is restricted to the vector injection site in the liver tumoral nodule (*arrows*). At later times the tracer accumulates in the bladder (*B*) and the intestines (*I*) owing to physiological elimination. In addition, this figure shows that higher vector titers do not necessarily produce higher transgene expression in the tumor as evidenced by [^{18}F]FHBG accumulation. **a,b** Coronal [^{18}F]FHBG PET images obtained from patients treated with 10^{12} viral particles (vp) (**a**) and with 2×10^{12} vp (**b**) starting 1.5 h and 6.5 h after injection of the tracer. For the patient treated with the lower adenoviral vector dose, specific [^{18}F]FHBG accumulation in the treated lesion can readily be ascertained in the early images while it can only be seen 6.5 h p.i. in the patient treated with the higher dose

site (see Figs. 4 and 5). Peñuelas et al. [72] observed that while intense accumulation of the radiotracer occurred in hepatic tumor nodules injected with AdCMVtk, the surrounding cirrhotic liver was spared. This finding is notable if we consider the marked hepatotropism of adenoviral vectors. It seems possible that the adenoviral particles which escape to the general circulation may not gain access to the hepatocytes in the context of liver cirrhosis owing to the fibrotic process and the capillarization of sinusoids, which may form a physical barrier preventing the infection of the cirrhotic liver. The observed sparing of non-tumoral cirrhotic tissue is a fortunate event since there is no risk of damaging normal hepatocytes via the effect of the prodrug. Thus, this finding argues in favor of the safety of gene therapy for this kind of tumor based on direct intratumoral injection of an adenoviral vector.

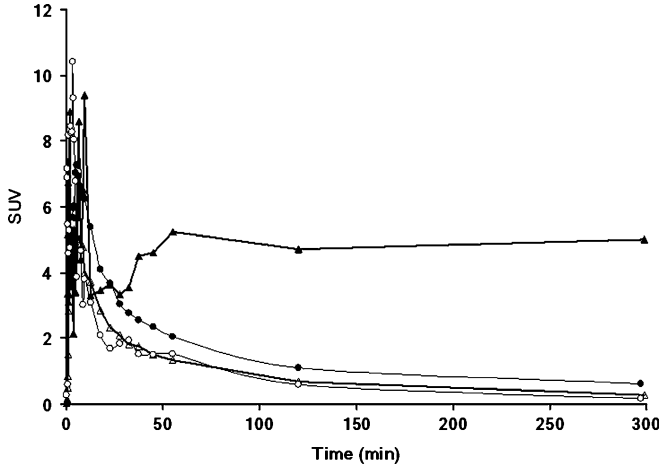

Fig. 7. [^{18}F]FHBG PET can provide very early images showing *HSV1-tk* transgene expression in humans. The graph illustrates the time course of the standardized uptake value (*SUV*) in two different areas of the liver: the treated tumor lesion (*triangles*) and the non-tumoral parenchyma (*circles*). For a patient treated with a low vector dose (*open symbols*), the two curves depict a similar pattern for both regions, while a clear difference between them can be perceived for a patient treated with a sufficiently high vector dose (*solid symbols*). A sufficiently high signal to background ratio can be obtained as soon as 50–60 min after tracer injection

In addition, PET whole-body imaging can provide very valuable information showing that the expression of the transgene is circumscribed to the treated lesion (Fig. 6) while no other organs—and especially the liver, spleen, lungs, heart, and testis—show specific accumulation of the tracer. However, the use of [^{18}F]FHBG or other metabolically similar tracers with hepatobiliary and renal clearance might impede the visualization of positively transduced tissues in the gastrointestinal and urinary tracts, and also in the brain, as the tracer does not cross the blood–brain barrier.

It is also of great importance to trace not only transgene expression but also vector biodistribution, as in many gene therapy protocols this is already a question of paramount importance, especially in relation to safety trials for new vectors. Schellingerhout et al. [117] used enveloped viral particles labeled with ^{111}In, allowing the viruses to be traced in vivo by scintigraphy. The labeling procedure did not significantly reduce the infectivity of the herpes simplex virus, and the virus did not release any significant amounts of the radionuclide within 12 h after labeling. Sequential imaging of animals after intravenous administration of the ^{111}In-virus showed fast accumulation in the liver and redistribution from the blood pool to liver and spleen. Also, when recombinant adenovirus serotype 5 knob (Ad5K) was radiolabeled with ^{99m}Tc [118] it retained specific, high-affinity binding to U293 cells, demonstrating that the radiolabeling process had no effect on the virus

capacity for receptor binding. In vivo dynamic imaging revealed that the liver binding followed an exponential rise to maximum, with a measured 100% extraction efficiency. The results of scintigraphy were confirmed in a biodistribution study. These data demonstrated that in vivo imaging may be a sensitive tool for measuring changes to liver tropism. Similar data were obtained for the adenoviral localization in the lungs [119]. These approaches could be used for labeling of the gene delivery vectors with a positron emitter and in vivo imaging with PET.

Evaluation of the necessary vector dose

In patients administered different adenoviral doses, we found that detectable tumor transduction was only observed when the dose of vector administered reached a certain threshold (Fig. 8). However, an increase in the vector dose above this threshold was not associated with enhanced [^{18}F]FHBG accumulation in the treated tumor. In fact, patients who received 10^{12} vp showed greater accumulation of the tracer in the tumor than those treated with 2×10^{12} vp. This variation in the accumulation of the radiolabel in the different neoplastic lesions treated may reflect dissimilarities in tumor biology leading to different transduction by adenoviruses or different ability of the tumor cells to transport the tracer. Alternatively, this phenomenon may be due to impaired gene expression when the cells are infected with an excess of viral particles or to differences in perfusion and shunting volume. These results further support the benefits of using an imaging study to measure the levels of gene expression directly, as it seems that it is not easy to predict them based exclusively on the dose of vector administered to the patient.

We found that the magnitude of [^{18}F]FHBG accumulation in the treated lesion varies from patient to patient and cannot be directly correlated with the adenoviral dose used in each particular case. Thus in a patient treated with a lower vector dose, the transduced tumor was visible at 1.5 h after [^{18}F]FHBG injection due to very active accumulation of the tracer in the neoplastic nodule (Fig. 6a) while in another case, despite a higher vector dose, the treated tumor was not readily visible at 1.5 h. In this case the transduced tumor only became apparent later (6.5 h), when the tracer cleared from the non-tumoral liver but remained trapped in the cells expressing *HSV1-tk* (Fig. 6b).

The ability of PET imaging to provide semi-quantitative data permits the pharmacokinetic analysis of the tracer in the treated tumor and in non-treated tissues. We have found that while in all patients who received a vector dose below a certain threshold, the SUV decreased with time in parallel in both tumor and non-tumor tissue, in patients who received 10^{12} or more vp, the SUV in the treated tumor remained high during the entire study period but decreased rapidly in the non-tumoral liver (Fig. 9). Accordingly, the ratio of radioactivity concentration in the tumor and non-tumor tissue increased steadily during the study period in the subjects treated with a sufficiently high adenovirus dose. In animal models, it has been demonstrated that for FHBG, better quantitation can be obtained when tracer kinetic modeling to quantitate rates of phosphorylation is applied [110].

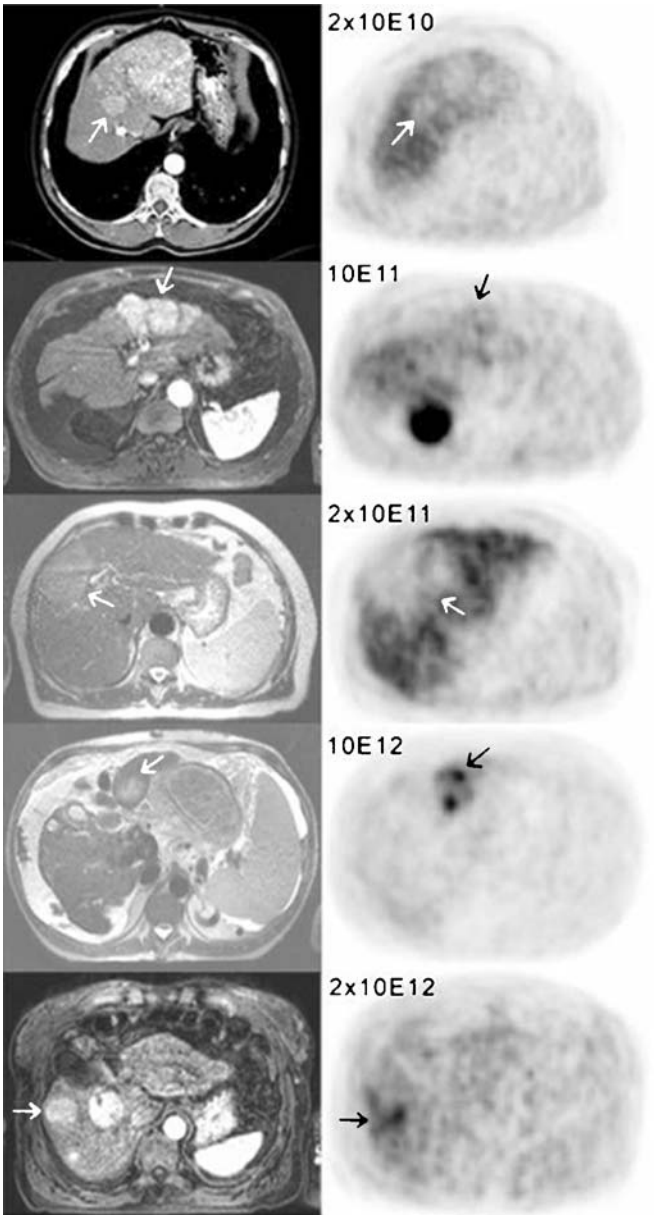

Fig. 8. Viral dose-dependent imaging of *HSV1-tk* transgene expression with PET. Transaxial anatomical images (MRI or CT) showing the treated tumor nodules and the corresponding [^{18}F]FHBG PET images obtained 60 min after injection of [^{18}F]FHBG in five patients (representative of five cohorts) enrolled to receive five consecutive adenoviral dose steps (2×10^{10}, 10^{11}, 2×10^{11}, 10^{12}, and 2×10^{12} vp, respectively). Patients received the vector by direct injection into the tumor nodule 2 days prior to imaging. Detectable tumor transduction can only be observed when the dose of vector administered reaches a certain threshold (10^{12} in this particular case). Diffuse accumulation of the tracer in non-tumoral hepatic tissue for the patients enrolled in the first three dose steps shows metabolic clearance of the tracer, while absent metabolism of the tracer in the tumor probably reflects cellular changes leading to dedifferentiation and loss of hepatocyte phenotype

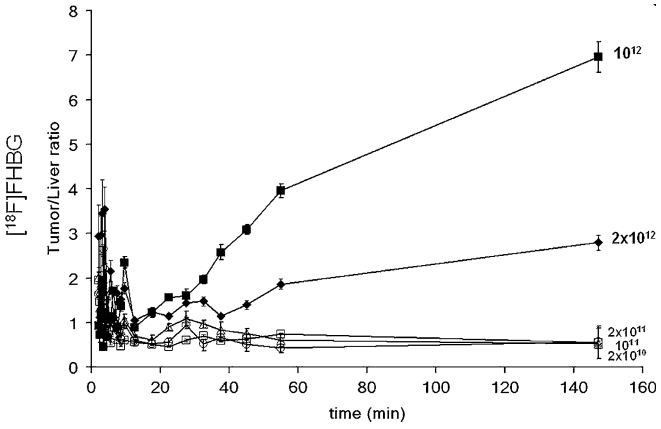

Fig. 9. PET imaging of transgene expression can provide semi-quantitative data that allow pharmacokinetic analysis of the tracer in the treated tumor and in non-treated tissues. The ratio of radioactivity concentration between the tumor and non-tumoral liver parenchyma increased steadily during the study period in the subjects treated with a sufficiently high adenovirus dose. As in Fig. 8, a representative case from each cohort of patients is shown. After 40 min, ratios were equal to or lower than 1 for the three lower doses. On the other hand, for higher vector doses, radioactivity was concentrated in the treated lesion due to *HSV1-tk* expression. *Error bars* show the error associated with the radioactivity ratios calculated by using the theory of error propagation

Duration of transgene expression

In a gene therapy procedure the duration of the effect of the genetic modification should be sufficiently long to achieve the desired therapeutic effect. Once more, there is very little information regarding how long the expression of a transgene lasts inside patient tissues. This information can also be obtained using PET. In hepatocarcinoma patients treated by suicide gene therapy with *HSV1-tk*, a [^{18}F]FHBG PET study performed at 9 days after the intratumoral injection of the vector showed no transgene expression in those patients who had shown positive tracer accumulation at just 2 days after tumor transduction (Fig. 10) [72]. These results indicate that the level of HSV1-TK expression was likely reduced to undetectable levels after this period of time. This was not a surprising finding since the tumor cells transduced with AdCMVtk are expected to be eliminated by the therapy with GCV and also by the immune response against the viral antigens expressed by the infected cells, although other possible explanations can be considered, such as loss of the genetic information which is episomal for adenoviral vectors. Yaghoubi et al. [70] state that the decrease in [^{18}F]FHBG accumulation after GCV treatment can be attributed to the following factors: (1) decrease in the level of HSV1-TK enzyme due to the elimination of transduced cells by GCV-induced cell death; (2) decrease in the level of HSV1-TK enzyme due to attenuated expression of the gene; (3) decrease in HSV1-TK enzymatic activity in vivo; (4) and decreased uptake of the tracer. Based on these data, we conclude that the major reason is in fact the decrease in HSV1-TK enzyme due to the elimination of transduced cells.

These results might permit better planning of the prodrug treatment schedule, and very early demonstration of the efficacy of the treatment. In this particular case in which transduced cells are hopefully killed by the GCV treatment, the results have to be carefully considered, but they undoubtedly serve as a proof of concept that the duration of transgene expression can be non-invasively monitored in humans by PET. Serially acquired PET studies in the same subject, when using a tracer labeled with a very short-lived isotope (such as [^{18}F]FHBG), are hence feasible and might provide valuable information for better gene therapy planning.

Once the duration of transgene expression is ascertained, one should consider the possibility of retreatment of the same individual with a new dose of the vector to maintain high levels of transgene expression and to try to kill any cells not eliminated by the first treatment.

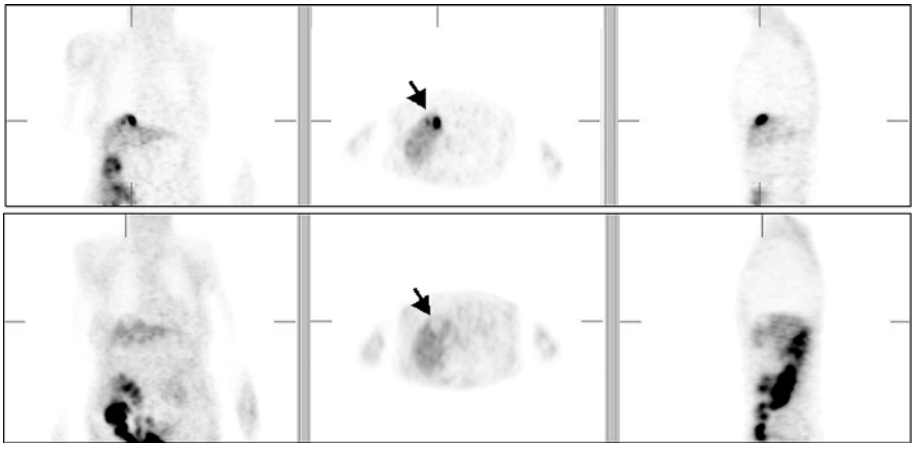

Fig. 10. Duration of transgene expression can be monitored by PET. [^{18}F]FHBG PET images were obtained 2 (*top row*) and 9 days (*bottom row*) after intratumoral injection of an *HSV1-tk*-encoding vector. Transgene expression can readily be observed at day 2 in the treated tumoral nodule (*arrow*), but not at day 9. GCV treatment (that started just after the first [^{18}F]FHBG PET study) was discontinued for at least 24 h before the second study in order to avoid competition between GCV and [^{18}F]FHBG. Lack of tracer accumulation by day 9 may have been due to several factors, as discussed in the text

Feasibility of tumor transduction after a second dose of the vector

It has been shown that PET can also be used to check this particular issue. In one patient who showed specific accumulation of [^{18}F]FHBG in the treated nodule after the first administration of AdCMVtk [72], we performed additional [^{18}F]FHBG PET studies after an additional dose of the vector 1 month later. This second dose of the vector was not followed by accumulation of [^{18}F]FHBG in the re-treated nodule (Fig. 11), a finding which may have been caused by several mechanisms. The first injection may have resulted in eradication of highly permissive hepatocellular carcinoma cells and selection of cells more resistant to adenoviral infection. Also, elicitation of a potent immune response after the first injection may have favored rapid clearance of the newly transduced cells after the second injection. But more probably, neutralization of the adenovirus by antibodies against the viral capsid strongly reduced the intensity of tumor transduction, as seen in animal models. In fact, in this patient the authors observed that the titer of neutralizing anti-adenovirus antibodies increased more than two logs after the first vector treatment (I. Peñuelas, unpublished results).

Improved vector design/protocol design

It is generally accepted that the Achilles' heel of gene therapy is the vectors utilized for gene delivery. Each new vector requires extensive safety testing before it is approved for human administration, and further testing once it has been used in humans. The overall process is not only very expensive, but also time consuming. Recent progress in vector production and multimodality imaging with optical/PET reporter genes will undoubtedly aid in the faster development of new vectors. Molecular imaging can help to trace not only transgene expression but also vector biodistribution, as discussed above.

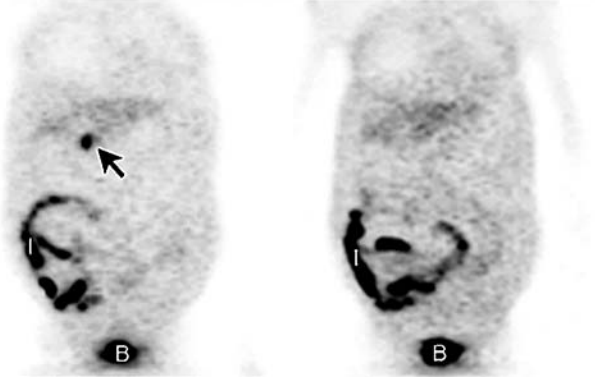

Fig. 11. PET imaging can show the feasibility of tumor transduction after a second dose of the vector. The benefits of double or multiple vector dose gene therapy protocols can be evaluated by [^{18}F]FHBG PET. Whole-body images are shown of the same patient at day 2 after the first dose of the vector (**a**) and 2 days after a second dose given 30 days after the first one (**b**). No transgene expression can be observed after the retreatment in the tumor nodule, while it is evident in the first treatment (*arrow*). Accumulation in the intestines (*I*) and bladder (*B*) can be seen in both cases due to physiological elimination of the tracer

Final considerations

Nearly all of the present knowledge regarding gene therapy comes from animal studies. However, the results obtained cannot be directly extrapolated to humans and many important questions remain unanswered. For each particular patient, there are some crucial questions that should be answered: Has targeted gene transfer been successful? Have any other tissues been transduced? Is the transferred gene expressed in sufficient extent and for sufficient duration? The availability of these data is critical for the clinical success of gene therapy, which relies on our ability to develop methodologies able to address these questions.

There is no doubt that the most exciting possibility for the use of PET in gene therapy is to monitor transgene expression. The availability of a quantitative, non-invasive tool to detect gene transfer and expression in vivo will probably change the way gene therapy protocols are designed and followed. Results with antitumoral *HSV1-tk*-based therapy in humans [72] have demonstrated the feasibility of such an approach. Much needs to be done in this field, as it is necessary to monitor not only the *HSV1-tk* gene, but any other therapeutic gene of interest that is amenable to use in a gene therapy protocol. Coupling of therapeutic and reporter genes may be one solution. However, it remains to be demonstrated which of the therapeutic gene to imaging reporter gene linking approaches is the best (fusion gene technology, bicistronic transcription units, bidirectional vectors, vector co-administration, etc.).

The uncertainties in clinical gene therapy are related not only to problems of gene delivery and gene expression but also to the lack of objective clinical endpoints to evaluate therapy response. In many cases the clinical benefit of the therapeutic procedure is hardly measurable or requires complex invasive procedures that give only a partial picture of the situation, as it is mostly based on molecular and histopathological analysis of biopsies. Hence, there is a real need for a technology that allows routine clinical use for a quantitative, whole-body spatiotemporal evaluation of gene delivery and expression as a way to determine and even predict the clinical outcome.

The conjunction of both of these ideas is of great practical importance. If molecular imaging by means of [^{18}F]FHBG PET can be used to predict therapy response and serially performed [^{18}F]FHBG PET studies can be used to monitor the effectiveness of the prodrug treatment in those cases in which response is foreseeable, we would have at our fingertips one of the most desired tools pursued by researchers in the field of suicide gene therapy. PET might show very early which patients must undergo the prodrug treatment, and in which ones this will be ineffective.

PET imaging, ideally combined with other molecular imaging modalities, can be used as an unsurpassable tool to aid in the design of improved vectors that would more

efficiently and precisely deliver the genes of interest to the target cells, and enhance our understanding of the molecular biology, biochemistry, genetics and pharmacokinetics of gene therapy vectors.

In summary, transgene expression can be monitored by PET in cancer patients. This non-invasive methodology represents a valuable tool for the assessment of gene expression in gene therapy in the clinical setting, and could be useful to define in a specific tissue or lesion the transduction efficiency of a given vector, to study transgene expression distribution, to determine its duration, and to help in the evaluation of new vectors and the design of novel therapeutic strategies.

Future perspectives and applications for imaging technologies in cancer gene therapy

Future studies will be influenced by the development of improved gene therapy vectors with higher transduction or transfection efficiencies and specificities. Development of new tracers with higher affinities for reporter proteins (enzymes, receptors or transporters) will improve the sensitivity of imaging. Furthermore, the use of mutants of *HSV1-tk* and perhaps other reporter genes may yield better imaging reporter gene/probe systems, which can help in imaging aimed at improving the gene therapy protocols. The transfer of nucleoside transporters can be used to enhance the tracer influx into the genetically modified cells and thereby increase suicide enzyme detection as well as therapeutic efficiency. The development of receptor-based reporter gene/probe systems with a lower background than the D_2R/[^{18}F]fluoroethyl-spiperone and using a commercially available ligand will increase their application in gene therapy clinical trials. Finally, reporter genes encoding proteins that are secreted into the blood, such as humanized *Gaussia* luciferase, provide an alternative to imaging or can complement imaging for the detection of the therapeutic gene [120].

Once the feasibility of gene expression imaging in humans has been demonstrated, we recommend that whenever possible molecular imaging be included in the protocols of gene therapy clinical trials. The use of different imaging surrogates would provide very valuable information on many different topics.

Indeed, it is very important to consider the possibility of including imaging in the protocol from the very beginning, when the trial is being designed. This would allow inclusion of both the desired therapeutic gene and the appropriate imaging reporter gene in the gene therapy vector prior to obtaining approval for the clinical trial from the regulatory agencies. Although this would require additional effort to determine the correlation between the expression of the two transgenes, imaging will yield essential information that would not otherwise have been gathered during the clinical trial. As shown in this review, the inclusion of an imaging reporter gene would permit not only monitoring of the time variation of transgene expression, but also non-invasive whole-body imaging of gene expression within and outside the target region, or even of the biodistribution of the vector on a whole-body basis and correlation with the sites of expression. The data obtained from the images would be of capital importance in a phase I trial, and could speed up considerably the approval of further phase II/III protocols.

In addition, molecular imaging should be considered as a way to monitor and evaluate response to the gene therapy procedure, or even as a prognostic indicator that would avoid unnecessary invasive sampling techniques and expensive and ineffective treatments.

The same principles as have been developed for gene therapy imaging can also be applied to cell-based therapies. Cells have been labeled in vitro with [^{18}F]FDG for short-term follow-up of cell trafficking [121] or with other longer-lived nuclides such as ^{64}Cu [122]. Nonetheless, such methods do not permit long-term follow-up of cell trafficking, a major issue than can be resolved by tracing cells in vivo with cell-specific labeled antibodies [123], or in a much better way by modifying the cells to carry an appropriate imaging reporter gene [109]. Monitoring of cell trafficking with genetically modified cells will permit grafting experiments and very long-term follow-up of the cell fate. For imaging of cell trafficking using reporter genes one must keep in mind the minimum number of cells that need to accumulate at one site in order to be detectable by PET, SPECT, or MRI as well as the possibility that the cells may shut down the expression of the reporter gene.

Finally, when the reporter and the therapeutic gene are different, one must consider the issue of reporter gene silencing even though the therapeutic gene is still being expressed and optimize the gene constructs so as to minimize the possibility of false negative results. False positive results by selective silencing of the therapeutic gene should also be taken into consideration.

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