Radiopharmaceuticals to Monitor the Expression of Transferred Genes in Gene Transfer Therapy

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SUMMARY. Gene transfer therapy is a new therapeutic modality that holds promise for effective treatment of disease by modification of the diseased cell's genetic structure. The genetic modification may involve replacement of a deficient or poorly-expressed normal gene, or the introduction of a foreign gene that will enhance the cell's sensitivity or resistance to specific therapeutic agents. Imaging provides the only realistic, non-invasive *in vivo* means by which to evaluate the success of the gene transfer, transcription and translation, and by which to detect unwanted transfer to non-target tissues. This paper briefly reviews the classical approaches to radiopharmaceutical design, and develops the concept of 'molecular' radiopharmaceuticals, with emphasis on gene therapy imaging. The Herpes simplex type-1 thymidine kinase (HSV-1 *tk*$^+$) gene, used initially as a suicide gene for gene therapy of glioma, is used as a model to focus the theme of scintigraphic imaging to monitor gene expression *in vivo*.

1. INTRODUCTION

Radiopharmaceuticals for *in vivo* scintigraphic imaging were initially used to obtain anatomical images based on physiological function that depended on blood flow, perfusion or clearance in a target region or organ. Early radiopharmaceuticals produced images attributable to vascular trapping (e.g. micro- and macro-aggregates) and reticulo-endothelial uptake (e.g. colloids), while others reflected physiological functions such as renal (e.g. iodo-aromatic acids and hydrophilic ^{99m}Tc-chelates) and hepatic (e.g. lipophilic ^{99m}Tc-chelates) clearance. (Table 1). From the beginning, there was substantial scientific interest in using radiotracers for imaging metabolic processes, including nucleoside antimetabolites directed against DNA synthesis. An example is the use of 5-iododeoxyuridine (IUDR), a thymidine analogue, to delineate tissues that have high mitotic indices (1). Modern imaging technologies, including x-ray CT and magnetic resonance imaging (MRI), have vastly superior spatial resolution compared to radiopharmaceutical scintigraphy (planar and tomographic imaging using single photon and positron emitters). It is the highly sensitive, quantitative functional measurements that provide the unique information that makes nuclear medicine imaging an invaluable diagnostic technique.

Table 1.Tracer accumulation mechanisms exploited in functional *in vivo* imaging (types of radiopharmaceutical are shown in parentheses).

- capillary blockage (aggregates)
- secretion / excretion (small molecules)
- phagocytosis (colloids, particulate & cellular immune reagents)
- diffusion / partition /pH (amines)
- receptor binding (steroids)
- protein binding (aptamers; non-specific binding agents)
- ionic substitution (anionic & cationic molecules & elements)
- transport (metabolic substrates)
- metabolic trapping (enzyme substrates)
- immune recognition (antibodies, fragments)
- nucleic acid binding (oligonucleotides)

The list of exploitable molecular targets for imaging has expanded immensely during the past two decades. Approaches to imaging molecular targets in membranes, and in extracellular, cytoplasmic and nucleoplasmic compartments, continue to exploit the sophisticated techniques developed in the basic sciences of molecular biology and genetic engineering.

In the 1970's, the new fields of molecular biology and genetic engineering created unanticipated opportunities for biomedical research. The immunological applications captured major attention in the early 1980's, and even today, a large, important component of experimental and clinical radiopharmaceutical science and nuclear medicine is based on radiolabelled monoclonal antibodies and monoclonal antibody fragments. Immunological research has stimulated strong therapy programs, including radiotherapy.

A number of fundamental barriers challenge the intracellular delivery of targeted, macromolecular radiopharmaceuticals (Table 2). Although many of these factors apply to all drugs, the macromolecules, especially the proteins, may also evoke an immunological response.

Table 2. *In vivo* barriers to the effective application of radiopharmaceuticals

- vascular and intracellular
  - * hydrolytic enzymes
  - * antibodies
  - * proteins (non-specific)
- lymphocytes
- reticuloendothelial cells
- membranes
  - * plasma
  - * nuclear & mitochondrial

Innovative *in vivo* delivery of 'biological' radiopharmaceuticals, whether they are monoclonal antibodies (MABs), MAB fragments, peptides or oligonucleotides remains a major challenge for scientists in this field. The *in vivo* instability of these substances, their frequently exaggerated (too fast or too slow) clearance kinetics and the difficulty in moving through biological membranes are among the challenges. The timely advent of solid phase and combinatorial synthetic chemistry has facilitated the move from these difficult to handle, large biomolecules to smaller, rigid chemical structures that are more 'tuneable'. Undoubtedly, although the molecular targets of today's macromolecular radiopharmaceuticals are unlikely to disappear, there is already a revival of small-molecule radiopharmaceuticals for diagnostic imaging.

Gene therapy represents the ultimate approach to the intracellular delivery of macromolecules. It by-passes the normal barriers to drug delivery, by genetically coding for the intracellular production of the desired substance in the target cell. Although this does not solve the problems of delivering a macromolecular radiopharmaceutical, it does pave the way for creation of unique molecular targets. The following paragraphs provide a brief review of the objectives and mechanics of gene therapy, after which some possible applications of imaging technologies are presented. A short **vocabulary** is included (Appendix A) as a guide for those less familiar with the field.

2. GENE THERAPY

Gene transfer therapy, directed *in situ* at the root-cause of disease within the host genome, has already been applied to a number of pathologies (Table 3). The first clinical trial in humans was started in 1990. The Recombinant DNA Advisory Committee of the US FDA has subsequently approved more than twenty gene therapy protocols that support upwards of 200 clinical trials world-wide. The current consensus is that the process needs substantial improvement, that there are risks and that there have not been any unequivocal cures (2).

Table 3. Applications of gene therapy.

- proliferative disease (e.g. atherosclerosis)
- enzyme deficiencies (e.g. ADA deficiency)
- infection (e.g. HIV)
- oncology (e.g. glioma)

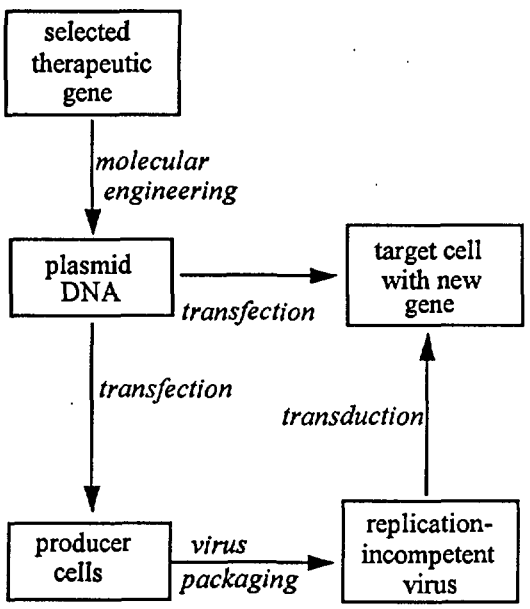
International efforts to map the human genome have greatly contributed to the identification of the genetic basis of many diseases. None-theless, there are major technical impediments to successful implementation of gene transfer, challenges like those inherent to classical intracellularly-targeted drugs (Table 2). Site-specific delivery, and control of gene catabolism, gene expression and gene deletion are major challenges that remain to be resolved.

Objectives of gene therapy have been classified (3) according to the transferred gene's intended role:

- gene replacement in hereditary diseases such as ADA deficiency and cystic fibrosis (e.g. enzyme replacement),
- gene replacement / supplementation for potentiation of host immune defences (e.g. cytokines) against cancer
- gene addition for drug resistance (e.g. multidrug resistance gene) to protect sensitive tissues such as the bone marrow, thereby permitting the use of otherwise toxic doses of a drug,
- gene addition for drug sensitivity (e.g. viral thymidine kinase gene to induce selective cell sensitivity to ganciclovir) in a cell that would normally be non-responsive to this drug, and,
- gene addition to disable abnormal DNA (e.g. HIV-DNA in host DNA is abnormal and can be treated as a genetic disease, this therapy would be directed towards T-cells).

Replacement gene therapy is particularly attractive for treating congenital disorders arising from absence or deficiency in expression of a gene, whereas introduction of new genes is a common objective in gene therapy of cancer. Importantly, current ethical considerations strictly limit human studies to somatic cell therapy.

Gene therapy begins with selection and isolation of the desired gene(s) from an eukaryotic, prokaryotic or viral donor. The naked gene is a charged strand of DNA strand that is readily degraded by circulating nucleases. This gene must be inserted into a delivery vector that will protect it and facilitate its movement through biological membranes and into the nucleus. A number of the obstacles to gene delivery are similar to the obstacles faced by other drugs (Table 2).The delivery options are outlined in Scheme 1; detailed discussion of the transfection vectors and the molecular engineering leading to a suitable plasmid are beyond the scope of this paper.



Scheme 1. Approaches to gene delivery to target cells.

There are several classes of vectors for gene delivery. Non-viral vectors have a large DNA-carrying capacity. They are non-immunogenic and non-infectious, but they are inefficient and non-selective in delivering the DNA to the target. Non-viral vectors include cationic liposomes, molecular conjugates, macro-molecular and ionic complexes (e.g. using lipids, charged carbohydrates and even inorganic salts) and the 'gene gun' which literally shoots the DNA into the cell.

The most popular and effective delivery vectors to date are retroviruses and adenoviruses that have been engineered to render them replication incompetent. Retroviruses insert their DNA directly into the DNA of dividing cells that they have invaded, but they have a limited DNA loading capacity (6-7 kb), and they transfect only actively dividing host cells. Adenoviruses carry larger DNA loads (15-25 kb) and are not cell cycle selective, but they do not actively insert their DNA into host DNA, thereby giving

rise to transient gene transfer. Because adenoviruses are cytoplasmic they are less likely to become infectious through the development of replication competency, but their immunogenecity raises an additional challenge to their use as vectors.

Gene transfer can be effected *in vitro* or *in vivo*. For *in vitro* transfer, the therapeutic gene is inserted into target cells (e.g. cord blood stem cells for a blood cell disease) isolated from the patient. After *in vitro* replication these homologous transformed or transfected cells are injected back into the donor patient at the appropriate tissue site. For *in vivo* delivery, the gene-carrying vector is injected (plasmid or engineered virus) or implanted (producer cell) into the target tissue *in situ*.

Interested readers are referred to reviews by Whartenby *et al* (3) and Anderson (4) for orientations to the literature.

3. GENE THERAPY IMAGING

Why use imaging in gene therapy?

When one considers the current methodologies of gene therapy, a number of uncertainties become apparent. Imaging is perhaps the only clinically-acceptable method available to deal with concerns related to delivery, transfer and expression of therapeutic somatic genes:

- *Site-specific delivery*: Systemic, but even stereotactic, administration of the vector creates the possibility of multiple, unwanted sites of transfer. Loss of efficacy and unexpected toxicity are the main consequences of delivery to non-target tissues.
- *Gene transfer*: From the viewpoint of clinical outcomes, gene expression will predicate successful treatment. However, transfer may be successful, but expression may be blocked. Knowledge of such occurrences are of critical importance in research leading to development of the proposed gene therapy protocol.
- *Expression*: If the transferred gene is not expressed, there can be no therapeutic benefit. Overexpression could present complications, depending on the role of the gene product.

These three issues can be addressed very explicitly by tissue biopsy together with immunohistochemical and nucleic acid analyses using the appropriate molecular probes. Unfortunately, biopsy is invasive and often not feasible because of the anatomical location and number of biopsies required. Also, biopsy provides discrete information relevant only to the biopsy sample itself, so that any heterogeneity in distribution may result in over- or under-estimation in the measurement. In sharp contrast, all sites are accessible simultaneously through imaging, and the signal volume-averaging properties insure that subject only to signal intensity, all sites will be sampled.

The utilization of a suicide gene as a reporter gene has an additional value in that there is no easy way to eliminate cells that proliferate as a result of gene transfer if there were to be a need to do so, other than with a suicide gene.

Clearly, the major role of imaging is to monitor the gene therapy protocol, alerting the therapist of impending risk, predicting therapeutic efficacy and indicating when no further improvement is to be expected.

4. CURRENT RESEARCH IN GENE THERAPY IMAGING

Two imaging modalities are currently of interest for gene therapy imaging. Beta-galactosidase has been investigated (5) as an enzyme target for Magnetic Resonance Imaging (MRI), and other targets such as tyrosinase are certain to emerge (6). Nuclear medicine approaches, using planar, SPET and PET imaging are the most common technologies at the present time, and will be discussed in greater detail in this paper. Current molecular targets for gene therapy imaging include therapeutic enzymes, receptors and nucleic acids encoded by the gene; of these, the enzyme-orientated projects are furthest advanced. There are three gene therapy enzyme-imaging projects that are based on the 'gene addition for drug sensitization' concept. *In vitro* and *in vivo* imaging of the herpes simplex virus

type-1 thymidine kinase (HSV-1 tk^+) has been the main enzyme target.

Virus encoded HSV-1 tk^+ was initially identified as an enzyme target for imaging herpesvirus infections, using radiolabelled nucleoside analogues (7-9) Selective phosphorylation in HSV-1-infected tissue, enhanced through intracellular metabolic trapping of phosphorylated metabolites formed the basis for this approach. The underlying principle, insertion of the viral gene (HSV-1 tk^+) into target tissues is now harnessed for suicide gene therapy. In essence, the only difference is that in viral disease the virus inserts its entire genome into that of the host, whereas in gene therapy, only the gene encoding for HSV-1 tk^+ is inserted (10). With the development of new gene expression targets (e.g. cytokines, receptors), it is likely that the suicide drug sensitizing gene will be replaced by other gene product targets. However, their roles as reporter genes remains of great interest. The radiopharmaceuticals used for their detection (enzyme substrates) are small, chemically-defined molecules that are highly diffusible and readily prepared, making them ideal for practical application in the clinic.

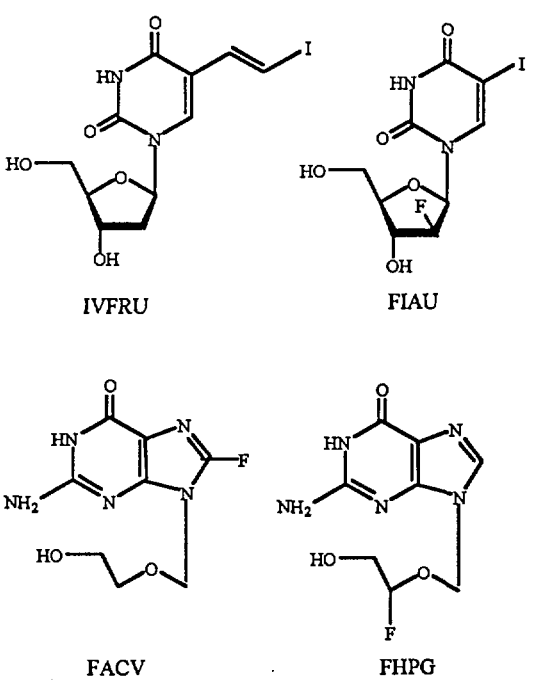
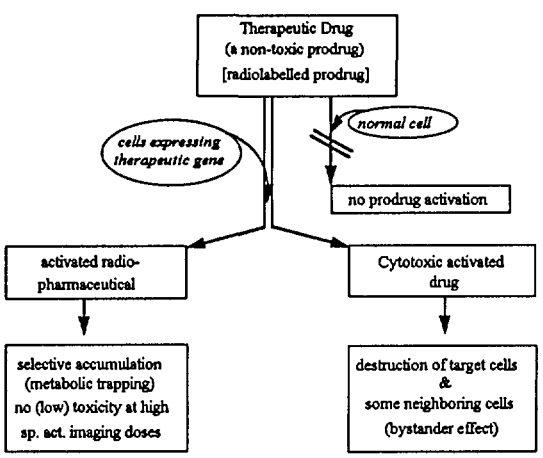


Figure 1. Nucleosides for imaging HSV-1 tk^+ expression in HSV-1 tk^+ gene therapy.

Current HSV-1 tk^+ -based gene therapy imaging studies are based on the radiotracers ^{123}I-IVFRU (11-13), ^{124}I-FIAU (14, 15), ^{18}F-FHPG (16) and ^{18}F-FACV (17) (Figure 1).

A second gene therapy enzyme target proposed was fungal cytosine deaminase using ^{18}F-5-FCT as the reporting radiopharmaceutical (18). This system is complicated by substrate catabolism and is therefore not expected to be viable with currently available radiopharmaceuticals. A bacterial reductase enzyme (19) for use in combination with the hypoxia imaging agent ^{123}I-IAZA (20) is another system that has been proposed recently (21).

These approaches are based on the use of genes that function as therapeutic suicide genes (negative selectable markers) when transferred into cells and combined with therapeutic drugs that are highly-specific substrates of the gene-encoded enzyme. The principles of suicide and reporter gene function are depicted in Scheme 2.



Scheme 2. Selective activation of prodrugs by enzymes encoded by suicide (reporter) genes, forming active drugs (radiopharmaceuticals) that are metabolically trapped.

The suicide gene product (e.g. HSV-1 tk^+) converts a non-toxic prodrug (e.g. IVFRU) into a toxic metabolite (e.g. IVFRU-5'-phosphate). Cells genetically modified to express these genes essentially commit metabolic suicide in the presence of the convertible prodrug. The prodrugs are essentially non-toxic before conversion, but highly toxic in cells that express the suicide gene. If metabolic conversion of the prodrug produces a polar or otherwise poorly

diffusible drug product, metabolic trapping is possible. If the prodrug is radioactive, then there will be an accumulation of radioactivity in the transformed cells, thus enabling imaging. Since unique metabolic pathways are seldom present in diseased cells, metabolic pathways present in bacteria, viruses or fungi have been exploited as targets.

5. CONCLUSIONS

Nuclear medicine has developed a focus on molecular targets, using both biological and chemical radiopharmaceuticals to obtain functional images. Gene therapy is a new treatment modality that can initiate or enhance the expression of unique molecular targets. Imaging these gene therapy targets will provide essential prognostic information critical to the assessment of both efficacy and untoward effects. Imaging based on the HSV-1 tk^+ gene, although still experimental, is the most advanced among three or four gene therapy imaging targets identified to date. For further information, a recent, detailed review (22) of gene therapy imaging is recommended.

6. ACKNOWLEDGEMENTS

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APPENDIX A. VOCABULARY

- *cloning vectors:* usually plasmid or bacteriophage, used to transport genes; may be amphotropic or ecotropic
- *expression:* cellular production of the gene-encoded product; see *transcription* and *translation*
- *gene transfer:* insertion of a new gene into a host cell; see *transduction* and *transfection*
- *plasmids:* small, autonomously replicating DNA molecules
- *reporter genes:* used to confirm successful transfer of a gene cassette
- *transcription:* synthesis of RNA from DNA
- *transduction:* acquisition and transfer of eucaryotic genes by retroviruses
- *transfection:* incorporation of the gene into the eukaryote cell via non-viral vectors
- *translation:* RNA-encoded protein synthesis
- *vectors* (*transfer vectors*): materials used to transfer the gene into the host cell.