Impact of Functional Genomics and Proteomics on Radionuclide Imaging

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The assessment of gene function following the completion of human genome sequencing may be performed using radionuclide imaging procedures. These procedures are needed for the evaluation of genetically manipulated animals or newly designed biomolecules, which requires a thorough understanding of physiology, biochemistry, and pharmacology. The experimental approaches will involve many new technologies, including in vivo imaging with single photon emission computed tomography and positron emission tomography. Nuclear medicine procedures may be applied for the determination of gene function and regulation using established and new tracers, or using in vivo reporter genes, such as genes encoding enzymes, receptors, antigens, or transporters. Visualization of in vivo reporter gene expression can be performed using radiolabeled substrates, antibodies, or ligands. Combinations of specific promoters and in vivo reporter genes may deliver information about the regulation of the corresponding genes. Furthermore, protein-protein interactions and activation of signal transduction pathways may be visualized noninvasively. The role of radiolabeled antisense molecules for the analysis of messenger ribonucleic acid (RNA) content has to be investigated. However, possible applications are therapeutic intervention using triplex oligonucleotides with therapeutic isotopes, which can be brought near to specific deoxyribonucleic acid sequences to induce deoxyribonucleic acid strand breaks at selected loci. Imaging of labeled siRNA makes sense if these are used for therapeutic purposes to assess the delivery of these new drugs to their target tissue. Pharmacogenomics will identify new surrogate markers for therapy monitoring, which may represent potential new tracers for imaging. Drug distribution studies for new therapeutic biomolecules are needed at least during preclinical stages of drug development. New treatment modalities, such as gene therapy with suicide genes, will need procedures for therapy planning and monitoring. Finally, new biomolecules will be developed by bioengineering methods, which may be used for the isotope-based diagnosis and treatment of disease.
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FUNCTIONAL INFORMATION is required after the identification of new genes to investigate the role of these genes in living organisms. This can be performed by analysis of gene expression, protein-protein interaction, or biodistribution of new molecules, and may result in new diagnostic and therapeutic procedures that include visualization of and interference with gene transcription and the development of new biomolecules to be used for diagnosis and treatment.

ASSESSMENT OF GENE EXPRESSION: IMAGING WITH ANTISENSE MOLECULES

The estimation of gene function using the tools of the genome program has been referred to as "functional genomics," which can be seen as describing the processes leading from a gene's physical structure and its regulation to the gene's role in the whole organism. Many studies in functional genomics are performed by the analysis of differential gene expression using high throughput methods, such as deoxyribonucleic acid (DNA) chip technology. These methods are used to evaluate changes in the transcription of many or all genes of an organism at the same time to investigate genetic pathways for normal development and disease. However, the assessment and modification of the messenger ribonucleic acid (mRNA) content of single genes is also of interest in functional studies.

Antisense RNA and DNA techniques have been originally developed to modulate the expression of specific genes. These techniques originated from studies in bacteria showing that these organisms are able to regulate gene replication and expression by the production of small complementary RNA molecules in an opposite (antisense) direction. Base pairing between the oligonucleotide and the corresponding target mRNA leads to highly specific binding and specific interaction with protein synthesis. Thereafter, several laboratories showed that synthetic oligonucleotides complementary to mRNA sequences could downregulate the translation of various oncogenes in cells.[1,2]

The silencing of genes also can be achieved by a mechanism that is based on double-stranded RNA (dsRNA). dsRNA is cleaved by a ribonucle-

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ase named Dicer to yield short RNA of 21 to 25 nucleotide length (siRNA). After interaction of these siRNA with a complex of cellular proteins to form an RNA-induced silencing complex (RISC), the RISC binds to the complementary RNA and inhibits its translation into a protein. This is known as RNA interference (RNAi) and can be used for treatment either by application of synthetic oligonucleotides or after introduction of DNA-bearing vectors that produce RNA hairpins in vivo, which are cleaved in the cell to the corresponding siRNA.[3-6]

Besides their use as therapeutics for specific interaction with RNA processing, oligonucleotides have been proposed for diagnostic imaging and the treatment of tumors. Assuming a total, human gene number between 24,000 and 30,000, calculations that consider alternative polyadenylation and alternative splicing result in a mRNA number between 46,000 and 85,000.[7] It is expected that an oligonucleotide with more than 12 (12-mer) nucleobases represents a unique sequence in the whole genome.[8] Because these short oligonucleotides can be produced easily, antisense imaging using radiolabeled oligonucleotides offers a high number of new tracers with high specificity. Prerequisites for the use of radiolabeled antisense oligonucleotides are ease of synthesis, stability in vivo, uptake into the cell, accumulation of the oligonucleotide inside the cell, interaction with the target structure, and minimal nonspecific interaction with other macromolecules. Nuclease resistance of the oligonucleotide, stability of the oligo-linker complex, and a stable binding of the radionuclide to the complex are required for the stability of radiolabeled antisense molecules. In this respect, modifications of the phosphodiester backbone, such as phosphorothioates, methylphosphonates, peptide nucleic acids, or gapmers (mixed backbone oligonucleotides), result in at least a partial loss in cleavage by RNAses.

Evidence has been presented of receptor coupled endocytosis as the low capacity mechanism by which oligonucleotides enter cells.[9,10] Subcellular fractionation experiments showed a sequestration of the oligonucleotides in the nuclei and the mitochondria of cervix carcinoma (HeLa) cells.[10] This phenomenon of fractionation, problems with in vivo stability of the oligonucleotides, as well as the stability of the hybrid oligo-RNA structures may prevent successful imaging of gene expression. Furthermore, binding to other polyanions, such as heparin based on charge interaction, result in unspecific signals.

However, successful antisense imaging has been reported in several studies. Accumulation of ^{111}In-labeled c-myc antisense probes with a phosphorothioate-backbone occurred in mice bearing c-myc overexpressing mammary tumors.[11] Imaging also was possible with a transforming growth factor, α-antisense oligonucleotide, an antisense phosphorothioate oligodeoxynucleotide for the mRNA of glial fibrillary acidic protein, and a ^{125}I-labeled antisense peptide nucleic acid targeted to the initiation codon of the luciferase mRNA in rat glioma cells permanently transfected with the luciferase gene.[12-15] Furthermore, positron emission tomography (PET) was used for the assessment of the biodistribution and kinetics of ^{18}F-labeled oligonucleotides.[16] In addition, ^{90}Y labeled phosphorothioate antisense oligonucleotides may be applied as targeted radionuclide therapeutic agents for malignant tumors.[17]

However, data obtained from mRNA profiling do not faithfully represent the proteome because the mRNA content seems to be a poor indicator of the corresponding protein levels.[18-20] The direct comparison of mRNA and protein levels in mammalian cells either for several genes in one tissue or for one gene product in many cell types revealed only poor correlations, with up to 30-fold variations. This might lead to misinterpretation of mRNA profiling results. Furthermore, mRNA is labile, leading to spontaneous chemical degradation as well as to degradation by enzymes that may be dependent on the specific sequence and result in nonuniform degradation of RNA. This phenomenon introduces quantitative biases that are dependent on the time after the onset of tissue stress or death. In contrast, proteins are generally more stable and show slower turnover rates in most tissues. A substantial fraction of interesting intracellular events is located at the protein level, operating primarily through phosphorylation/dephosphorylation and the migration of proteins. Also, proteolytic modifications of membrane-bound precursors appear to regulate the release of a large series of extracellular signals, such as angiotensin or tumor necrosis factor.

Because protein levels often do not reflect mRNA levels, antisense imaging may not be a generally applicable approach for a clinically use-

ful description of biological properties of tissues. Expression profiling data would be more useful if mRNA samples could be enriched for transcripts that are being translated.[21] This can be achieved by fractionation of cytoplasmic extracts in sucrose gradients, which leads to the separation of free ribonucleoprotein particles (mRNP) from mRNA in ribosomal preinitiation complexes and from mRNA loaded with ribosomes (polysomes). Because only the polysomes represent actively translated transcripts, this fraction should be directly correlated with de novo synthesized proteins. To date, polysome imaging with nuclear medicine procedures has not been tried or may not even be possible. Therefore, antisense imaging for the determination of transcription by hybridization of the labeled antisense probe to the target mRNA makes sense in cases in which RNA and protein content are highly correlated. Successful imaging was possible in cases in which the expression of the protein was proven or the gene of interest was introduced by an expression vector.[11-15] In the absence of such a correlation between mRNA and protein content, the diagnostic use of antisense imaging seems questionable. Therapeutic applications may use triplex oligonucleotides with therapeutic isotopes, such as Auger electron emitters, which can be brought near to specific DNA sequences to induce DNA strand breaks at selected loci. The imaging of labeled siRNA makes sense if these are used for therapeutic purposes to assess the delivery of these new drugs to their target tissue.

ASSESSMENT OF GENE REGULATION BY REPORTER ASSAYS

Functional assessment by gene expression analysis may be performed by a comparison of sequences near coding regions in diverged organisms, assuming that nucleotides conserved in noncoding regions between these pairs of organisms identify functional sites that typically are response elements for regulatory proteins. Among genes that show correlated expression patterns across a large variety of biological conditions, a significant fraction is expected to be coregulated (ie, responsive to common expression factors). However, the prediction of promoter locations and properties, as well as analyses for the precise identification of intron/exon architecture and boundaries of gene transcripts still are hampered by unacceptable uncertainties.[22,23]

With the increasing availability of intrinsically fluorescent proteins that can be genetically fused to virtually any protein of interest, their application as fluorescent biosensors has extended to dynamic imaging studies of cellular biochemistry, even at the level of organelles or compartments participating in specific processes.[24] On the supracellular level, fluorescence imaging allows the determination of cell-to-cell variation, the extent of variation in cellular responses, and the mapping of processes in multicellular tissues. Furthermore, visualization of intracellular gradients in enzymatic activities, such as phosphorylation and guanosine triphosphatase activity, can now be related to morphogenetic processes in which the distribution of activity mirrors the cellular response.

However, for the examination of whole organisms, and especially organ systems in deeper parts of the body, in vivo reporter systems are promising. Biological systems are more complex than cell cultures because external stimuli may affect and trigger cells. Therefore, noninvasive dynamic in vivo measurements are needed to study gene regulation in the physiologic context of complex organisms. These in vivo reporters may be used also for the characterization of promoter regulation involved in signal transduction, gene regulation during changes of the physiologic environment, and gene regulation during pharmacologic intervention. This may be performed by combining specific promoter elements with an in vivo reporter gene. Furthermore, the functional characterization of new genes will result in new diagnostic targets and, possibly, also in new tracers for their visualization, which may be substrates for enzymes or transporters, ligands for receptors, or antibodies for antigens. However, there may be concerns about the image resolution, even when animal scanners are used. Therefore, autoradiography or fluorescence resonance energy transfer represents alternatives in cases in which high sensitivity or resolution at the micrometer range is required.

ASSESSMENT OF GENE FUNCTION: STUDIES IN ANIMALS BEARING MUTATIONS

Usually, producing a knockout mutation or altering the expression of a gene results in phenotypes that provide insights into the function of specific genes. Besides these genotype-driven mu-

Table 1. Genes and Radioistope Imaging Methods Used for The Monitoring of Successful Gene Transfer

Gene	Principle	Imaging Method	Tracer/Contrast Agent
Enzymes:			
CD	Enzyme activity	MRS, PET	5-Fluorocytosine
HSVtk	Therapeutic effects	MRI, MRS, PET, SPECT	FDG, HMPAO, misonidazole
HSVtk	Enzyme activity	SPECT, PET	Specific substrates
HSVtk mutant	Enzyme activity	PET	Specific substrates
Tyrosinase	Metal scavenger	MRI, SPECT, scintigraphy	In
Nonsuicide reporter genes:			
SSTR2	Receptor expression	SPECT, scintigraphy	Radiolabeled ligand
D2R	Receptor expression	PET	Radiolabeled ligand
Transferrin receptor	Receptor expression	MRI	Radiolabeled ligand
CEA antigen	Antigen expression	Scintigraphy	Radiolabeled antibody
Modified green fluorescence protein	Transchelation	SPECT, scintigraphy	^{99m}Tc-glucoheptonate
Human sodium iodide transporter	Transport activity, therapy	Scintigraphy	^{131}I
Human norepinephrine transporter	Transport activity, therapy	Scintigraphy	^{131}I-MIBG

Abbreviations: CD, cytosine deaminase; CEA, carcinoembryonic antigen; D2R, dopamine-2 receptor; FDG, ^{18}F-fluorodeoxyglucose; HMPAO, hexamethyl propyleneamine oxime; ^{131}I-M$_1$BG, ^{131}I-meta-iodobenzylguanidine; MRS, magnetic resonance spectroscopy; SPECT, single photon emission computed tomography; SSTR, somatostatin receptor.

tations, there is an increasing need for phenotype-driven mutations, such as those obtained with the alkylating agent N-ethyl-N-nitrosurea, to identify genes that are involved in specific kinds of disease. This approach needs no assumptions regarding which genes and what kinds of mutations are involved in a particular phenotype or disease. To maximize the efficiency of the related experiments, it will be necessary to develop multiple assays working at different levels of description (eg, morphologic, physiologic, biochemical, or behavioral) to detect a large number of different phenotypes in a given set of mutagenized mice. The screening of a mutagenized population is usually performed with phenotypically visible coat color markers in combination with selection genes as neomycin resistance gene and the herpes simplex virus thymidine kinase (HSVtk) gene. However, using genes such as HSVtk or others in combination with scintigraphic imaging as noninvasive in vivo reporters may be an attracting alternative.

Measurement of Gene Regulation and Gene Transfer–In Vivo Reporter Genes

In vivo reporter genes are genes that can be visualized noninvasively using either radionuclide based methods, magnetic resonance imaging (MRI), or methods based on the detection of fluorescence, luminescence. Genes encoding for enzymes, receptors, antigens, and transporters have been used. Enzyme activity can be assessed by the accumulation of the metabolites of radiolabeled specific substrates, receptors by the binding, and internalization of ligands, antigens by binding of antibodies and transporters by the uptake of their substrates (Table 1). Because expression of the HSVtk gene leads to phosphorylation of specific substrates and to the accumulation of the resulting negatively charged metabolite, this gene can be used as an in vivo reporter gene.[25-39] A general problem with this gene is that the affinity of these specific substrates for the nucleoside transport systems as well as for the enzyme is rather low, which may be a limiting factor for cellular accumulation. Therefore, at present, the ideal tracer for HSVtk imaging has not been identified, and more efforts have to be directed at synthesizing radiolabeled compounds with improved biochemical properties. A mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk) has been used as an in vivo reporter gene for PET to improve the detection of low levels of PET reporter gene expression.[40] The successful transfer of this mutant gene resulted in an enhanced uptake of the specific substrates [8-^{3}H]penciclovir, and 8-[^{18}F]fluoropenciclovir in C6 rat glioma cells, with a 2-fold increase in accumulation compared with wild type HSVtk-expressing tumor cells.

Two receptor genes have been used to visualize genetically transformed tumors. The dopamine D2 receptor gene is an endogenous gene that is not likely to invoke an immune response. Furthermore, the corresponding tracer 3-(2′-[^{18}F]-fluoroethyl) spiperone (FESP) rapidly crosses the blood brain barrier, can be produced at high specific activity, and is currently used in patients. The tracer uptake

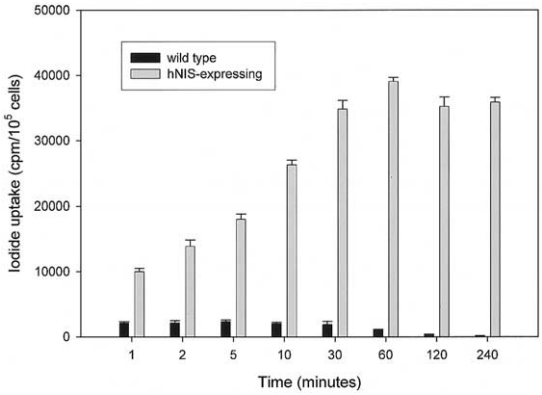

Fig 1. Time dependence of $^{125}I^-$ uptake in wild type Morris hepatoma cells and in human sodium iodide symporter (hNIS)-expressing cells. Mean values and standard deviation (n = 3).

in nude mice after transfection with an adenoviral-directed hepatic gene delivery system and also in transplanted stable tumor cells was proportional to in vitro data of hepatic FESP accumulation, dopamine receptor ligand binding, and the D2 receptor mRNA.[41] Tumors modified to express the D2 receptor retained significantly more FESP than wild type tumors. In modified, nonsmall, cell lung cell lines expressing the human type 2 somatostatin receptor and transplanted in nude mice, a 5 to 10-fold higher accumulation of a ^{99m}Tc or ^{188}Re labeled somatostatin-avid peptide was obtained.[42]

The low expression of tumor-associated antigens on target cells for radioimmunotherapy may be encountered by the transfer of the specific gene. Therefore, the gene for the human carcinoembryonic antigen (CEA) was transfected in a human glioma cell line, resulting in high levels of CEA expression.[43] In these modified tumor cells, high binding of an ^{131}I-labeled CEA antibody was observed in cell culture experiments as well as by scintigraphic imaging.

Transchelation of oxotechnetate to a polypeptide-motif from a biocompatible complex with a higher dissociation constant than that of a dicglycilcysteine complex has been used to obtain binding of oxotechnetate to synthetic peptides and recombinant proteins, like a modified green fluorescence protein.[44] In these experiments, intramuscular injection of synthetic peptides bearing a diglycylcysteine motif resulted in accumulation of radioactivity after application of ^{99m}Tc-glucoheptonate. Peptides with 3, metal-binding diglycylcysteine motifs showed a 3-fold higher accumulation as compared with the controls. This principle can be applied also to recombinant proteins that appear at the plasma membrane.[45]

The gene of the human sodium iodide symporter (hNIS) has been transferred in a variety of tumor models.[46-59] The corresponding protein seems promising because, besides iodine, it accepts pertechnetate, which is commonly available as a substrate. Transfer of the hNIS gene into tumor cells caused a significant increase in iodide uptake by a factor of 84 to 235. Animal studies with wild type and hNIS-expressing tumors in rats showed a maximum uptake after 1 hour, and a continuous disappearance of the radioactivity out of the body as well as of the hNIS-expressing tumors (Figs 1 and 2).[49,50,55] Although the NIS activity is asymmetrical favoring iodide influx, there is obviously an efflux activity with the consequence that in cells that do not organify iodide, the concentration of intracellular iodide will decrease proportionally to the external iodide concentration. However, the hNIS gene may be used together with ^{121}I, ^{124}I or even with ^{99m}Tc-pertechnetate as a simple reporter system for the visualization of other genes in bicistronic vectors, which allow coexpression of 2o different genes.

In comparison to the other reporter genes described, the sodium iodide symporter gene may present the advantage that it is not likely to interact with underlying cell biochemistry and that its substrate pertechnetate is widely available. Iodide is not metabolized in most tissues, and, although sodium influx may be a concern, no adverse effects have been observed to date.[60] The HSVtk gene may alter the cellular behavior towards apoptosis by changes in the deoxynucleotide (dNTP) pool,[61] antigens may cause immunoreactivity, and receptors may result in second messenger activation,

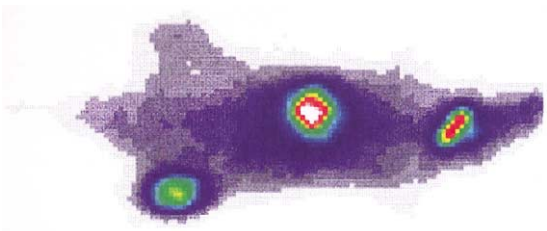

Fig 2. Scintigraphic image of a tumor bearing male Copenhagen rat subcutaneously transplanted with human sodium iodide symporter (hNIS)-expressing (right thigh) or wild type prostate adenocarcinoma cells (left thigh) at 4 hours after injection of $^{131}I^-$. (Color version of figure is available online.)

such as triggering signal transduction pathways. These possible interactions have to be studied in detail in future experiments. For the dopamine 2 receptor system, a mutant gene has been applied that shows the uncoupling of signal transduction.[62]

Protein-Protein Interaction

Protein interaction analysis delivers information about the possible biological role of genes with unknown function by connecting them to other better characterized proteins. Furthermore, it detects novel interactions between proteins that are known to be involved in a common biological process and also novel functions of previously characterized proteins. Protein interactions have been deduced by purely computational methods or using large scale approaches.[63,64] Ideally, the characterization of protein interactions should be based on experimentally determined interactions between proteins that are known to be present at the same time and in the same compartment. With the increasing availability of intrinsically fluorescent proteins that can be genetically fused to a wide variety of proteins, their application as fluorescent biosensors has extended to dynamic imaging studies of cellular biochemistry, even at the level of organelles or compartments participating in specific processes.[65] Fluorescence imaging allows the determination of cell-to-cell variation, the extent of variation in cellular responses, and the mapping of processes in multicellular tissues. In addition, procedures for noninvasive, dynamic in vivo monitoring are needed to show whether the protein interactions also work in the complex environment of a living organism, such as mice, rats, or humans, where external stimuli may affect and trigger cells or organ function. The yeast 2-hybrid technique has been adapted for in vivo detection of luciferase expression using a cooled charge coupled device (CCD) camera.[66] GAL4 and VP16 proteins were expressed separately, and associated by the interaction of MyoD and Id, 2 proteins of the helix-loop-helix family of nuclear proteins that are involved in myogenic differentiation. In this experimental setting, association of GAL4 and VP16 resulted in expression of firefly luciferase, which was under the control of multiple copies of GAL4 binding sites and a minimal promoter.

Drawbacks of the cooled CCD camera are mainly its limitation to small animals with different efficiencies of light transmission for different organs, the lack of detailed tomographic information, and lack of an equivalent imaging modality applicable to human studies.[67] Therefore, another approach based on the 2-hybrid system used a fusion of a mutated HSVtk gene and the green fluorescent protein gene for in vivo detection of the interaction between p53 and the large T antigen of simian virus 40 by optical imaging and PET. Interaction of both proteins resulted in association of GAL4 and VP16 and reporter gene expression, which was visualized after administration of a ^{18}F labeled specific substrate for HSVtk.[68]

Current approaches are based on intracistronic complementation and reconstitution by protein splicing. Complementation does not require the formation of a mature protein. Both parts of the reporter protein are active when closely approximated.[69] The complementation strategy can be exploited for a wide range of studies directed at determining whether proteins derived from 2 active genes are coincident or colocalized within cells. Other applications include transgenic animals expressing complementary lacZ mutants from 2 promoters of interest, which should identify cell lines in which the products of both genes coincide spatially and temporally. Reconstitution is based on protein splicing in trans, which requires the re-association of an N-terminal and C-terminal fragment of an intein, each fused to split N- and C-terminal halves of an extein, such as a reporter gene like enhanced green fluorescent protein or luciferase.[70] Re-associated intein fragments form a functional protein-splicing active center, which mediates the formation of a peptide bond between the exteins, coupled to the excision of the N- and C-inteins. Newer strategies fuse the intein segments to interacting proteins, which results in initiation of protein splicing in trans by protein-protein interaction.[71]

The feasibility of imaging interaction of MyoD and Id based on both a complementation and a reconstitution strategy has been shown using a CCD camera and split reporter constructs of firefly luciferase.[72] After cotransfection of 2 plasmids, the complementation as well as the reconstitution strategy achieved activities between 40% and 60% of the activity obtained after transfection of a plasmid bearing the full length reporter gene. A cooled CCD camera was applied for visualization of luciferase activity in nude mice. This strategy presents a promising tool for the in vivo evaluation

of protein function and intracellular networks, and may be extended to approaches involving combinations of reporter genes and radionuclides. However, MyoD and Id are strong interacting proteins. There may be a weaker in vivo signal when systems with a weaker interaction are used.

Protein interactions occur not only as physical interactions but also as functional interactions. These may be studied by the analysis of promoters or promoter modules[73] or using combinations of specific promoters and reporter genes.[60,74] For the examination of whole organisms, in vivo reporter systems are promising. These in vivo reporters may be used for the characterization of promoter regulation involved in signal transduction, gene regulation during changes of the physiologic environment, and gene regulation during pharmacologic intervention. This may be performed by combining specific promoter elements with an in vivo reporter gene. However, specific promoters are usually weak.[75] This problem was addressed using a 2-step amplification system for optical imaging of luciferase and PET imaging of HSVtk expression.[76] In that study, tissue specific reporter gene expression driven by the prostate specific antigen promoter was enhanced by the transfer of a plasmid bearing a GAL4-VP16 fusion protein under the control of the prostate specific antigen promoter, together with a second plasmid bearing multiple GAL4 responsive elements and the reporter gene. Optical imaging revealed a 5-fold signal enhancement in nude mice. Another strategy may be the use of multiple specific enhancer elements upstream of their corresponding promoter.

GENE TRANSFER: VISUALIZATION OF VECTOR DISTRIBUTION

An understanding of the biodistribution of vectors carrying therapeutic genes to their targets would be helpful to develop strategies for the target-specific delivery of therapeutic agents. This has been studied using radiolabeled cells as well as radiolabeled viral vectors. The feasibility of cell-based "suicide gene" therapy using the bystander effect associated with the HSVtk-expressing ovarian cancer cell line PA1-STK was assessed in malignant pleural mesothelioma in which the homing of genetically modified cells has been followed in 4 patients. HSVtk-expressing ovarian cancer cells that were labeled with ^{99m}Tc and infused into the pleural space localized preferentially to the tumor at the chest wall.[77] Also, the biodistribution of hepatocytes labeled with ^{111}In-oxine was assessed in rats to optimize hepatocyte transplantation systems for ex vivo gene therapy or liver repopulation. Transplanted cells were successfully localized by scintigraphic imaging. The scintigraphic patterns of cell distribution were different when hepatocytes were transplanted via the spleen or the internal jugular vein, which deposit cells into separate vascular beds.[78] Using dextran-coated, superparamagnetic iron oxide particles derivatized with a peptide sequence from the human immunodeficiency virus-tat protein, Josephson and coworkers were able to detect labeled cells by MRI.[79] They suggested that this technique may have applications for the MRI based tracking of cells in the body.

Schellingerhout and coworkers used enveloped viral particles labeled with ^{111}In, which allowed the detection of virus distribution in vivo by scintigraphy.[80] The labeling procedure did not significantly reduce the infectivity of the herpes simplex virus without a significant release of the radionuclide within 12 hour 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, recombinant adenovirus serotype 5 knob was radiolabeled with ^{99m}Tc and retained specific, high-affinity binding to U293 cells, showing that the radiolabeling process had no effect on the virus capacity for receptor binding.[81] In vivo dynamic imaging revealed that the liver binding followed an exponential increase to maximum, with a measured 100% extraction efficiency. The results of scintigraphy were confirmed in a biodistribution study.

MEASUREMENT OF GENE TRANSFER DURING GENE THERAPY WITH SUICIDE GENES

We may expect a better understanding of the mechanisms of carcinogenesis, tumor progression, and the patient's immune response from basic science. Furthermore, the characterization of tumor cell-specific properties allows the development of new treatment modalities, such as gene therapy. Some of these new approaches include the transfer of foreign genes into normal or tumor tissue: (1) protection of normal tissues (as the bone marrow), which are normally targets for cytotoxic drugs.

Table 2. Suicide Genes Used for Treatment

Enzyme	Prodrug	Active Drug
E coli purine nucleoside phosphorylase (DeoD)	6-Methylpurine-2′-deoxyribonucleoside	6-methylpurine
E coli thymidine phosphorylase	5′-Deoxy-5′-fluorouridine, tegafur	5-fluorouracil
E coli guanosine-xanthine phosphoribosyltransferase (gpt)	6-thioxanthine, 6-thioguanine	6-thioxantine-MP, 6-thioguanine-MP
Xanthine oxidase	Xantine, hypoxanthine	H_2O, OH and O_2 radicals
Carboxypeptidase G2	Benzoic acid mustards-glumatic acid	Benzoic acid mustards
Alkaline phosphatase	Etoposide phosphate, doxorubicin phosphate, mitomycin phosphate	Etoposide, doxorubicin, mitomycin phenol mustard
Cassava linamarase	Linamarin	Aceto cyanohydrin, HCN
Carboxypeptidase A	Methotrexate-alanine	Methotrexate
Cytosine deaminase	5-Fluorocytosine (5FC)	5-Fluorouracil (5FU)
Cytosine deaminase + uracil phosphoribosyltransferase	5-Fluorocytosine	5-Fluorouracil + 5-fluorouridine-5′monophosphate
Penicillin amidase	Doxorubicin-phenoxyacetamide	Doxorubicin
	Melphalan-phenoxyacetamide	Melphalan
	Palytoxin-4 Hydroxyphenoxyacetamide	Palytoxin
β-glucosidase	Amygdalin	Cyanide
β-glucoronidase	Epirubicin-glucoronide, phenol mustard-glucuronide, daunomycin-glucoronide, adrimycin-glucoronide	Epirubicin, phenol mustard, daunomycin, adriamycin
β-Lactamase	Phenylenediamine mustard cephalosporin	Phenylenediamine mustard
E coli nitroreductase	CB1954 (5-aziridin 2,4-dinitrobenzamidine)	5-Aziridin 2,4-hydroxyamino 2-nitrobenzamidine
Cytochrome P450 2B1	Cyclophosphamide	Phosphoramide mustard
Rabbit hepatic carboxylesterase	irinotecan	SN38 (7-ethyl-10-hydroxycamptothecin)
Human deoxycytidine kinase	Cytosine arabinoside (Ara C), fludarabine	Ara-CMP, fludarabine-MP
Dm-deoxyribonucleoside Kinase	Pyrimidine and purine analogs	Phosphorylated metabolites
HSV thymidine kinase	Ganciclovir (GCV), aciclovir (AVC)	Phosphorylated metabolites
VZV thymidine kinase	6-methoxypurine arabinonucleoside (araM)	Phosphorylated metabolite

This may be achieved by the transfer of the gene for the drug efflux pump glycoprotein P; (2) improvement of the host antitumor response by increasing the antitumor activity of tumor infiltrating immune competent cells or by modifying the tumor cells to enhance their immunogeneity. This approach relies on the introduction of genes that are responsible for the production of foreign surface antigens or of genes, which lead to local production and secretion of cytokines; (3) reversion of the malignant phenotype either by suppression of oncogene expression or by introduction of normal tumor suppressor genes. The inactivation of oncoproteins may be performed by introduction of genes for intracellular antibodies (intrabodies) against these oncogenes or by the use of antisense oligonucleotides and ribozymes; and (4) direct killing of tumor cells by the transfer of cytotoxic or prodrug-activating genes (Table 2).[30,82]

Noninvasive tools are needed to evaluate the efficiency of gene transfer in terms of gene transcription for the clinical application of these new treatment modalities. In that respect, nuclear medicine procedures offer a high sensitivity in the picomolar range. Labeling of substrates with radioactive isotopes and administration of very small amounts of these tracers allow the assessment of biochemical or physiologic processes without any interference with the phenomena to be studied.

Expression of the herpes simplex virus thymidine kinase (HSVtk) has been studied in several tumor models after viral as well as nonviral transfer of the gene. The principle of in vivo HSVtk imaging was first shown by Saito and coworkers

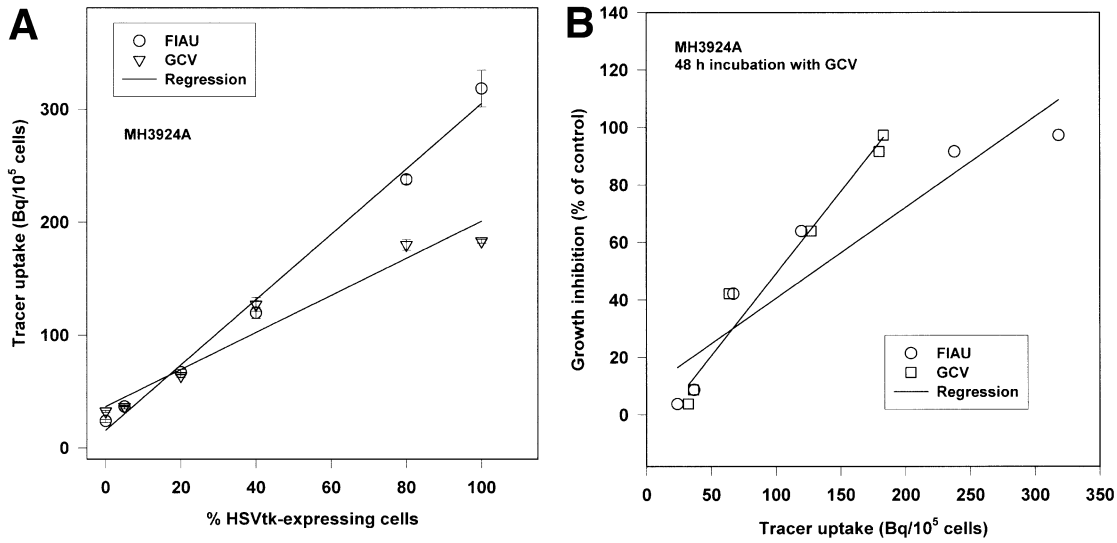

Fig 3. (A) Uptake of the specific substrates ganciclovir (GCV) and 5-iodo-2′-fluoro-2′deoxy-1-b-D-arabinofuranosyluracil (FIAU) in different mixtures of control cells and herpes simplex virus thymidine kinase (HSVtk)-expressing Morris hepatoma cells after 4-hour incubation. The tracer uptake and the amount of HSVtk-expressing cells were correlated with $r = 0.97$ and $r = 0.99$, respectively. (B) Relation of total GCV and FIAU uptake after 4-hour incubation with the tracer and the growth inhibition after 48 hours exposure to 5 μM GCV. The tracer uptake and the growth inhibition in HSVtk-expressing Morris hepatoma cells were correlated with $r = 0.98$ and $r = 0.94$, respectively.

for the visualization of HSV encephalitis.[36] In all studies, the enhanced uptake of specific substrates such as 5-iodo-2′-fluoro-2′deoxy-1-b-D-arabinofuranosyluracil (FIAU), fluorodeoxycytidine (FCdR), 5-fluoro-1-(2′-deoxy-fluoro-β-D-ribofuranosyl) uracil (FFUdR), ganciclovir, 8-[^{18}F]fluoroganciclovir (PGCV), 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine ([^{18}F]FHBG), and 9-[(3-^{18}F-fluoro-1-hydroxy-2-propoxy)methyl]-guanine ([^{18}F]-FHPG) was seen in genetically modified tumor cells in vitro and in vivo.[25-39] Furthermore, ganciclovir, FFUdR, and the FIAU uptake were highly correlated to the percentage of HSVtk-expressing cells and to the growth inhibition as measured in bystander experiments (Fig 3).[28,37,83] In rats infected with adenovirus particles there was also a significant positive correlation between the percent injected dose of 8-[^{18}F]fluoroganciclovir FGCV retained per gram of liver and the levels of hepatic HSVtk expression.[27]

To elucidate the transport mechanism of the specific HSVtk substrate ganciclovir inhibition/competition experiments were performed in rat hepatoma and human mammary carcinoma cells. The nucleoside transport in mammalian cells is known to be heterogeneous with 2 classes of nucleoside transporters: the equilibrative, facilitated diffusion systems and the concentrative, sodium-dependent systems. During these experiments, competition for all concentrative nucleoside transport systems and inhibition of the ganciclovir transport by the equilibrative transport systems were observed, while the pyrimidine nucleobase system showed no contribution to the ganciclovir uptake.[28,29] In human erythrocytes, acyclovir has been transported mainly by the purine nucleobase carrier.[84] Due to a hydroxymethyl group on its side chain, ganciclovir has a stronger similarity to nucleosides and, therefore, may be transported also by a nucleoside transporter. Moreover, the 3′hydroxyl moiety of nucleosides was important for their interaction with the nucleoside transporter.[85]

In rat hepatoma cells as well as in human mammary carcinoma cells, the GCV uptake was much lower than the thymidine uptake.[28,29] Therefore, in addition to low infection, efficiency of the current viral delivery systems slow transport of the substrate, and also its slow conversion into the phosphorylated metabolite is limiting for the therapeutic success of the HSVtk/GCV system. Cotransfection with nucleoside transporters or the use of other substrates for HSVtk with higher affinities for nucleoside transport and phosphorylation by HSVtk may improve therapy outcome.

In human glioblastoma cells, the effects of cytosine deaminase (CD) gene transfer were evaluated. When exposed to ^{3}H-fluorocytosine (5-FC),

these cells produced ^{3}H-5-FU, while in the control cells, only ^{3}H-5-FC was detected.[86] Moreover, significant amounts of 5-FU were found in the medium of cultured cells, which may account for the bystander effect observed in previous experiments. However, uptake studies revealed a moderate and nonsaturable accumulation of radioactivity in the tumor cells and lack of inhibition by hypoxanthine or uracil, suggesting that 5-FC enters the cells only via diffusion. Although a significant difference in 5-FC uptake was seen between CD-positive cells and controls after 48 hours incubation, no difference was observed after 2 hours of incubation. Furthermore, a rapid efflux could be shown. Therefore, 5-FC transport may be a limiting factor for this therapeutic procedure, and quantitation with PET has to rely rather on dynamic studies and modeling, including high-performance liquid chromatography analysis of the plasma, than on nonmodelling approaches.[86] To evaluate the 5-FC uptake in vivo, a rat prostate adenocarcinoma cell line was transfected with a retroviral vector bearing the *Escherichia coli* CD gene. The cells were sensitive to 5-FC exposure but lost this sensitivity with time. This result may be due to inactivation of the viral promoter (cytomegalovirus) used in this vector. In vivo studies with PET and ^{18}FC showed no preferential accumulation of the tracer in CD-expressing tumors, although high-performance liquid chromatography analysis revealed a production of 5-fluorouracil, which was detectable in tumor lysates as well as in the blood of the animals.[87]

A functional analysis of bacterial CD and yeast CD expressed in COS-1 cells showed that both recombinant enzymes used cytosine with equal efficacy, but 5-FC was an extremely poor substrate for the bacterial CD, with an apparent catalytic efficiency 280-fold lower than that observed for the yeast CD.[88] Furthermore, after retroviral infection of tumor cell lines with the different genes, the IC50 of 5-FC was 30-fold lower in yeast CD-infected cells than in cells with expression of the bacterial CD gene. In subcutaneous human colorectal carcinoma xenografts in nude mice in vivo magnetic resonance spectroscopy was performed to measure yeast CD transgene expression in genetically modified tumors by direct detection of CD-catalyzed conversion of 5-fluorocytosine to 5-fluorouracil.[89] A 3-compartment model revealed a first-order kinetics, suggesting that the yeast CD was not saturated in vivo in the presence of measured intratumoral 5-FC concentrations higher than the in vitro determined affinity (K_m) values.

RADIONUCLIDE THERAPY IN GENETICALLY MODIFIED TUMORS

Currently used viral vectors for gene therapy of cancer have a low infection efficiency, leading to moderate or low therapy effects. This problem could be solved using an approach that leads to accumulation of radioactive isotopes with β-emission. In this case, isotope trapping centers in the tumor could create a cross firing of β-particles, thereby efficiently killing transduced and nontransduced tumor cells. Currently, the transfer of genes for sodium iodide or norepinephrine transporters or the thyroid peroxidase has been tried.

The first step in the complex process of iodide trapping in the thyroid is the active transport of iodide together with sodium ions into the cell that is mediated by the sodium-iodide symporter. This process against an electrochemical gradient requires energy, is coupled to the action of Na+/K+-adenosinetriphosphatase, and is also stimulated by thyroid-stimulating hormone.[90-94] Since the cloning of the human and rat complimentary (c)DNA sequences, several experimental studies have been performed that investigated the recombinant expression of the hNIS gene in malignant tumors by viral transfer of the hNIS gene under the control of different promoter elements.[46-59,95,96] Although all of them reported high initial uptake in the genetically modified tumors, differing results have been obtained concerning the efficiency of radioiodine treatment based on NIS gene transfer, with generally very high doses given to tumor-bearing mice.

In vitro a rapid efflux of iodide occurred with 80% of the radioactivity released into the medium after 20 minutes.[49,50,53,55-57,97] Because the effectiveness of radioiodine therapy depends not only on the type and amount but also on the biological half-life of the isotope in the tumor, a therapeutically useful absorbed dose seems unlikely for that type of experiment. A significant efflux was also seen in vivo when doses were applied that are commonly administered to patients: only 0.4 ± 0.2 (1,200 MBq/m^2) and $0.24 \pm 0.02\%$ (2,400 MBq/m^2) of the injected dose per gram in the hNIS-expressing tumors were observed at 24 hours after tracer administration.[50] Similarly, Nakamoto and

coworkers found less than 1% of the injected radioactivity at 24 hour after ^{131}I administration in modified MCF7 mammary carcinomas, although initially a high uptake was seen.[53] This corresponds to a very short half-life of ^{131}I (approximately 7.5 hour) in rat prostate carcinomas, which also has been described by Nakamoto and coworkers for human mammary carcinomas with a calculated biological half-life of 3.6 hour. In contrast, differentiated thyroid carcinoma showed a biological half-life of less than 10 days and normal thyroid of approximately 60 days.[98]

However, in vitro clonogenic assays revealed selective killing of NIS-expressing cells in some studies.[46,47,52,58] Also, bystander effects have been suggested in 3-dimensional spheroid cultures.[47] In vivo experiments in stably transfected human prostate carcinoma cells showed a long biological half-life of 45 hours.[58] This resulted in a significant tumor reduction (84% ± 12%) after a single intraperitoneal application of a very high ^{131}I dose of 111 MBq.[58,59] The investigators concluded that the transfer of the NIS gene causes effective radioiodine doses in the tumor and might, therefore, represent a potentially curative therapy for prostate cancer. To improve therapy outcome, Smit and coworkers investigated the effects of low iodide diets and thyroid ablation on iodide kinetics.[57] The half-life in NIS-expressing human follicular thyroid carcinomas without thyroid ablation and under a regular diet was very short, with 3.8 hours. In thyroid ablated mice that were kept on a low iodide diet, the half-life of radioiodide was increased to 26.3 hours, which may be due to the diminished renal clearance of radioiodine and lack of iodide trapping by the thyroid. Subcutaneous injection of 74 MBq in thyroid-ablated nude mice that were kept on a low-iodide diet postponed tumor development. However, 9 weeks after therapy, tumors had developed in 4 of the 7 animals. The estimated tumor dose in these animals was 32.2 Gy.[57]

However, these studies used very high doses: in a mouse 74 and 111 MBq correspond to administered doses of 11,100 and 16,650 MBq/m^2, respectively. This is far more than the doses used in patients. In rat prostate carcinomas, treatment with amounts of ^{131}I corresponding to those given to patients (1,200 and 2,400 MBq $^{131}I/m^2$) resulted in only 3 Gy absorbed dose in the genetically modified tumors.[50] Because approximately 80 Gy have been described as necessary to achieve elimination of metastases in patients with thyroid cancer, this is not likely to induce a significant therapeutic effect in the tumors.[24] Furthermore, the experiments were performed under ideal conditions with 100% NIS expressing cells in the tumors. Given the low infection efficiency of currently viral vectors in vivo, the absorbed dose in a clinical study would be considerably lower. There are also other differences in these studies (ie, tracer administration, time of treatment, and animal and tumor models). Therefore, differences in the biodistribution of iodide and the biochemical properties of the tumor cells may lead to differences in iodide retention.

To prolong the iodine retention time in tumors, some investigators tried to transfer simultaneously the NIS and the thyroperoxidase gene.[46,97,100] Boland and coworkers observed iodide organification in cells coinfected with both the NIS and the thyroid peroxidase (TPO) gene in the presence of exogenous hydrogen peroxide.[46] However, the levels of iodide organification obtained were too low to increase significantly the iodide retention time. In a variety of different cell lines including human anaplastic thyroid carcinoma and rat hepatoma cells, we were not able to measure TPO enzyme activity or enhanced accumulation of iodide regardless of very high amounts of hTPO protein after the retroviral transfer of the human TPO gene.[100] In contrast, Huang and coworkers observed an increased radioiodide uptake (by a factor of 2.5) and retention (by a factor of 3), and enhanced tumor cell apoptosis after transfection of nonsmall cell lung cancer cells with both human NIS and TPO genes.[97] However, a 72% efflux occurred in vitro during the first 30 minutes, indicating a very low hTPO activity in the genetically modified cells. Therefore, other modulations of iodide retention in tumor cells should be evaluated in future studies.

Lithium has reduced the release of iodine from the thyroid and, therefore, was used to enhance the efficacy of radioiodine treatment of differentiated thyroid cancer.[101] When the biological half-life was less than 3 days, lithium prolonged the effective half-life by more than 50%.[101] In FRTL-5 rat thyroid cells and in primary cultures of porcine thyroid follicles, 2 mmol/L lithium suppressed thyroid-stimulating hormone-induced iodide uptake, iodide uptake stimulated by 8-bromo-cAMP, iodine organification, and de novo thyroid hor-

mone formation.[102,103] Lithium is concentrated by the thyroid, and inhibits thyroidal iodine uptake, iodotyrosine coupling, alters thyroglobulin structure, and inhibits thyroid hormone secretion.[102-106] Therefore, if enhanced iodide trapping in the thyroid by lithium relies on interaction with iodine coupling to tyrosine residues or inhibition of thyroid hormone secretion, an organification process is still needed to obtain a sufficient iodine accumulation in the tumor. First experiments in our laboratory showed no significant effect of lithium in hNIS-expressing hepatoma cells.[55]

A further option to increase therapy outcome is the use of biologically more effective isotopes. Dadachova and coworkers compared ^{188}Re-perrhenate with ^{131}I for the treatment of NIS-expressing mammary tumors.[107] In a xenografted breast cancer model in nude mice, ^{188}Re-perrhenate showed NIS-dependent uptake into the mammary tumor. Dosimetry showed that ^{188}Re-perrhenate delivered a 4.5-times higher dose than ^{131}I and, therefore, may provide enhanced therapeutic efficacy. Furthermore, the high LET-emitter astatine-211 has been suggested as an isotope with high radiobiologic effectiveness.[53,108] First experiments showed that the tracer uptake in NIS-expressing cell lines increased up to 350-fold for ^{123}I, 340-fold for $^{99m}TcO_4^-$, and 60-fold for ^{211}At. Although all radioisotopes showed a rapid efflux, higher absorbed doses in the tumor were found for ^{211}At, as compared with ^{131}I.[108] In conclusion, a definitive proof of therapeutically useful absorbed doses in vivo after the transfer of the NIS gene is still lacking. Further studies have to examine pharmacologic modulation of iodide efflux or the use of the hNIS gene as an in vivo reporter gene.[50,60]

Another approach of a genetically modified isotope treatment is the transfer of the norepinephrine gene. ^{131}I-meta-iodobenzylguanidine (MIBG), a metabolically stable false analogue of norepinephrine, has been widely used for imaging and targeted radiotherapy in patients with neural crest derived tumors, such as neuroblastoma or pheochromocytoma. In the adrenal medulla and in pheochromocytoma, MIBG is stored in the chromaffin neurosecretory granules.[109] The transport of MIBG by the human norepinephrine transporter (hNET) seems to be the critical step in the treatment of MIBG-concentrating tumors. The mechanism of MIBG uptake, which is qualitatively similar to that of norepinephrine, has been studied in a

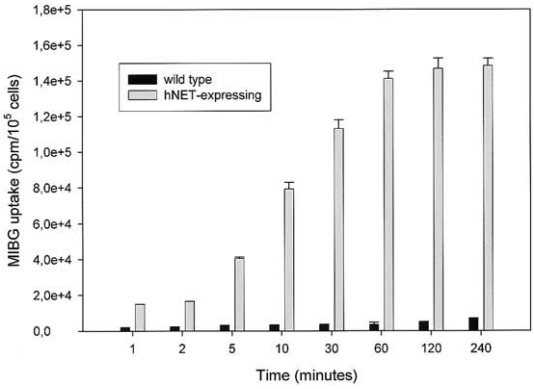

Fig 4. Time dependence of ^{131}I-meta-iodobenzylguanidine (MIBG) uptake in wild-type Morris hepatoma cells and in human norepinephrine transporter (hNET)-expressing cells. Mean values and standard deviation (n = 3).

variety of cellular systems, and 2 different uptake systems have been postulated. Although most tissues accumulate MIBG by a nonspecific, nonsaturable diffusion process, cells of the neuroadrenergic tissues and in malignancies derived thereof show an active uptake of the tracer that is mediated by the noradrenalin transporter.[110-113] The clinical use of the MIBG radiotherapy is so far restricted to neural crest derived malignancies and due to insufficient ^{131}I-MIBG uptake, therapy in these patients with a tumor is not curative.

The effect of hNET gene transfection was investigated in a variety of cells, including COS-1 cells, HeLa cells, glioblastoma cells, or rat hepatoma cells, and a 3-fold to 36-fold increase of ^{131}I-MIBG or noradrenaline accumulation was achieved (Fig 4).[112,114-116] In vivo experiments performed with nude mice bearing both the hNET expressing and the wild type tumor showed a 10-fold higher accumulation of ^{131}I-MIBG in the transfected tumors, with respect to the wild type tumors. Furthermore, in rat hepatoma cells when compared with previous studies concerning the efflux of ^{131}I from hNIS-expressing cells,[49] a longer retention of MIBG in the hNET-transfected cells was observed.[116] Nevertheless, 4 hours after incubation with MIBG, an efflux of 43% of the radioactivity was determined for the recombinant cells, while wild type cells had lost 95% of the radioactivity. In view of a MIBG radiotherapy in nonneuroectodermal tumors, an intracellular trapping of the tracer is required to achieve therapeutically sufficient doses of radioactivity in the genetically modified tumor cells. In that respect, a positive correlation has

been observed between the content of chromaffin neurosecretory granules and the uptake of radiolabeled MIBG.[117]

Human glioblastoma cells transfected with the bovine NET gene were killed by doses of 0.5 to 1 MBq/mL ^{131}I-MIBG in monolayer cell culture as well as in spheroids.[115] Accordingly, the investigators expected the intratumoral activity in a 70 kg patient to be 0.021%. This corresponds to the range of MIBG uptake usually achieved in neuroblastoma. However, data obtained from in vitro experiments cannot be applied to the in vivo situation. In contrast to stable in vitro conditions, the radioactive dose delivered to the tumor in vivo differs due to decreasing radioactivity in the serum and due to heterogeneity within the tumor tissue. To calculate the radiation dose in a particular tumor more precisely, an in vivo dosimetry is superior. Using 14.8 MBq ^{131}I-MIBG for the application in tumor-bearing mice corresponding to 2,200 MBq/m^2 in humans, a radiation dose of 605 mGy in the hNET-expressing and of 75 mGy in the wild type tumor was calculated.[116] With regard to the treatment of patients with a nonneuroectodermal tumor transfected by the hNET gene, this absorbed dose is too low to evoke any tumor response. In addition, as with most gene transfer studies, the in vivo experiments were performed with animals that had been transplanted with 100% stable hNET-expressing cells. Therefore, due to the low in vivo infection efficiency of virus particles, infection of tumor cells in vivo will result in even lower radiation doses.

Future development should comprise pharmacologic modulation of MIBG retention or interaction with competing catecholamines. The use of the recombinant hNET gene product as an in vivo reporter is not promising because the images showed a high background and relatively faint appearance of the genetically modified tumor.[116] Finally, it has been speculated whether the transfer of the NET gene into pheochromocytoma or neuroblastoma cells may enhance the efficiency of MIBG therapy.[115]

RADIOISOTOPE IMAGING FOR THE MONITORING OF NEW THERAPEUTIC MODALITIES

Through the accumulation of genomics and proteomics data, novel biomolecules may be discovered or designed. This process can be performed by directed genome evolution, metabolic pathway engineering, protein engineering, analyses of functional genomics and proteomics, high throughput screening techniques, and the development of bioprocess technology.[118] The products of this process will be monoclonal antibodies, vaccines, enzymes, antibiotics, therapeutic peptides, and others. The design of a biocatalyst involves 2 main steps, which can be iterative: (1) making a set of mutant biocatalysts and (2) searching the set of mutants with the desired properties. In this stage of development, isotope based methods are needed to assess binding characteristics of antibodies or ligands, or measure new transport or enzyme functions in vivo. Furthermore, in later stages, the coupling of antibodies or peptides with α or β emitters may be used for therapeutic purposes.

Based on the assumption that many therapeutic drugs act through mechanisms involving perturbations of protein expression, an efficient drug could be defined as one that restores the expression levels of a cell or an organ to the normal state.[119] Therefore, measurement of the pattern of protein changes can be used to describe the mechanism of action. Proteomics offers the opportunity to obtain complimentary information to genomic-based technologies for the identification and validation of protein targets in following time-dependent changes in protein expression levels that result from selective interaction with specific biological pathways and identifying protein networks (functional proteomics). Changes in protein expression or function could also serve as targets for noninvasive imaging procedures. In transgenic mice bearing in vivo reporter genes, protein interactions may be monitored in native organs and tissues during treatment, as well as during development. Intracellular signaling has been visualized in vitro with combinations of specific regulatory elements (eg, promoters, enhancers) and reporter genes, such as the secreted alkaline phosphatase downstream of several copies of specific transcription factor binding sequences.[120] A similar approach may be developed for in vivo detection by use of in vivo reporter genes.

By defining key changes in protein content or function, it may be possible to use radiolabeled ligands or substrates to assess therapeutic effects on specific parts of the proteome. This process may be performed using established tracers for new therapies or using new tracers that have been

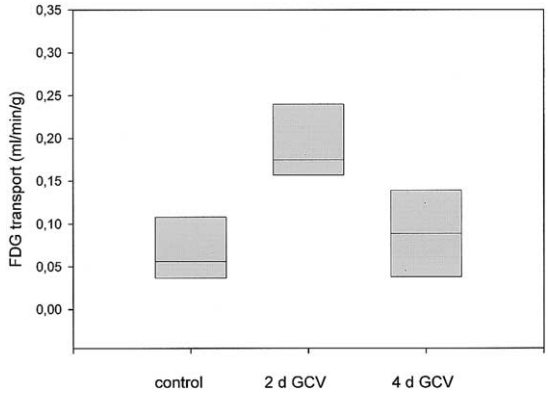

Fig 5. ^{18}F-fluorodeoxyglucose (FDG) uptake in untreated animals (n = 8), as well as after 2 (n = 7) and 4 days (n = 5) of treatment with 100 mg GCV/kg bw. Changes in the FDG transport rate (K_1).

identified either by functional studies of new genes, or by the analysis of changes in expression or functional patterns by the high-throughput methods of functional genomics. To date, most studies have been performed to assess the effects of suicide gene therapy. The monitoring of gene therapy with suicide genes has been performed by measurements of tumor perfusion and tumor metabolism. Tumor perfusion in ganciclovir (GCV) treated HSVtk-expressing tumors measured after intravenous administration of [^{99m}Tc] hexamethylpropyleneamine oxime, increased by 206% at day 2 after the onset of ganciclovir treatment.[121] Also, 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 ganciclovir treatment.

The ^{18}F-fluorodeoxyglucose (FDG) uptake has been shown to be a useful and very sensitive parameter for the evaluation of glucose metabolism during or early after the treatment of malignant tumors. Dynamic PET measurements of ^{18}FDG uptake in rats bearing HSVtk-expressing hepatomas revealed an uncoupling of FDG transport and phosphorylation with enhanced transport values and a normal phosphorylation rate after 2 days of GCV treatment (Fig 5).[122,123] These tumors showed a significant increase of the glucose transporter 1, as shown by immunohistochemistry.[124] The increase in FDG transport normalized after 4 days, while the phosphorylation rate increased. As an 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. Consequently, inhibition of glucose transport by cytochalasin B or competition with deoxyglucose increased apoptosis.[124]

Besides these established, monitoring procedures, we may expect new biochemical pathways emerging from proteomics research, leading to the use of radiolabeled substrates for enzymes, transport systems, specific structures on cell membranes, or the detection of apoptosis. For the in vivo detection of apoptosis, mainly 2 targets in the apoptotic pathway are of interest: (1) the presentation of phosphatidylserine residues at the outer side of the plasma membrane and (2) the appearance of activated caspases.[125,126] Phosphatidylserine is maintained at the inner site of the plasma membrane by the adenosine triphosphate-dependent enzymes floppase and translocase.[127] Apoptosis induced inactivation of these enzymes, and activation of a scramblase leads to the appearance of phosphatidylserine on the outer side of the membrane. This effect has been used recently to develop an imaging agent for apoptosis: Annexin V, a 35 kD human protein with a high affinity for cell membrane bound phosphatidylserine, was labeled with ^{99m}Tc and investigated for its uptake in apoptotic cells.[128,129] An increased accumulation was found in Jurkat cells, where the programmed cell death was initiated by growth factor deprivation, anti-CD95 antibody, and doxorubicin treatment. Also, anti-CD95 treated mice showed a 3-fold increase in hepatic ^{99m}Tc-Annexin V accumulation in response to severe liver damage with histologic evidence of apoptosis. Finally, increased uptake was detected in animal models using the acute rejection of transplanted heterotopic cardiac allografts or transplanted murine B cell lymphomas treated with cyclophosphamide.[129]

Because caspases play a key role during the early period of the intracellular signal cascade of cells undergoing apoptosis, benzyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethyl ketone (Z-VAD-fmk), a pan-caspase inhibitor, was evaluated as a potential apoptosis imaging agent.[130] Uptake measurements were performed with Morris hepatoma cells, which showed expression of the HSVtk gene. Apoptosis was induced by the treatment of the cells with ganciclovir, and a 2-fold increase of [^{131}I]I-Z-VAD-fmk uptake was found at the end of treatment with the HSVtk/suicide system, which constantly remained increased for

the following 4 hours. The slow cellular influx and lack of uptake saturation of [^{131}I]IZ-VAD-fmk are evidence for simple diffusion as transport mechanism. In addition, the absolute cellular uptake of [^{131}I]IZ-VAD-fmk was low. Instead of using an inhibitor, synthetic caspase substrates are currently investigated, which may accumulate in the apoptotic cell by metabolic trapping, thereby enhancing the imaging signal. Besides these established monitoring procedures, we may expect new biochemical pathways emerging from proteomics research, leading to the use of radiolabeled substrates for enzymes, transport systems, or specific structures on cell membranes.

DESIGN OF NEW BIOMOLECULES FOR RADIOISOTOPE BASED DIAGNOSIS AND THERAPY

Two techniques are important for the design of new biomolecules: (1) DNA shuffling and (2) phage display libraries. DNA shuffling mimics natural recombination by allowing in vitro homologous recombination of DNA.[131,132] Therefore, a population of related genes is randomly fragmented, and subjected to denaturation and hybridization, followed by the extension of 5′ overhang fragments by *Taq*DNA polymerase. A DNA recombination occurs when a fragment derived from one template primes a template with different sequences. The applications of this method include the improvement of enzyme properties, development of altered metabolism pathways, antibiotics and pharmaceutical proteins, development of plasmids or viruses for novel vaccines, and gene therapy applications. Genes from multiple parents and even from different species can be shuffled in a single step in operations that do not occur in nature but may be very useful for the development of diagnostic and therapeutic approaches.

The principle of phage-displayed peptide libraries is the display of the peptide libraries fused with the carboxy-terminal domain of the minor coat protein, gene III protein fragment, on the surface of a filamentous phage. The relevant molecule is then directly detected and screened using the target molecules and amplificated after infection of *E coli*. This allows a rapid selection (within weeks) of particular clones from large pools ($> 10^{10}$ clones), and determination of the amino acid sequence of a peptide displayed on a phage by sequencing the relevant section of the phage genome. This technique has been used for searching antibodies, receptors for new drug discovery and cancer therapy, either as an antagonist or an agonist of a natural ligand-receptor interaction,[133,134] and custom-made enzymes for gene therapy.

Many new molecular structures have been cloned and will be available as potential novel diagnostic or drug discovery targets. The target selection and validation will become the most critical component in this process. The evaluation of genetically manipulated animals or newly designed biomolecules will require information about physiology, biochemistry, and pharmacology, and the experimental approaches will apply many technologies, including in vivo imaging with single photon emission computed tomography and PET.[135,136] Nuclear medicine procedures can be applied for the determination of gene function and gene regulation using established or new tracers to study effects in knockout mice or in transgenic animals. The measurement of gene regulation may also be performed using in vivo reporter genes, such as enzymes, receptors, antigens, or transporters. Pharmacogenomics will identify new surrogate markers for therapy monitoring, which may represent potential new tracers for imaging. Also, drug distribution studies for new biomolecules are needed to get drug approval in preclinical stages of drug development. Finally, bioengineering will lead to the design of new biomolecules by methods such as DNA shuffling or phage display procedures, which may be used for new approaches in the isotope-based diagnosis and treatment of disease.

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