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In Vivo Molecular-Genetic Imaging

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Abstract Noninvasive in vivo molecular-genetic imaging has developed over the past decade and involves nuclear (PET, gamma camera), magnetic resonance, and in vivo optical imaging systems. Although three different imaging strategies — ''direct,'' ''indirect'' and ''surrogate'' — are being used, most current in vivo molecular imaging strategies are ''indirect'' and involve the coupling of a ''reporter gene'' with a complimentary ''reporter probe.'' Imaging the level of probe accumulation provides indirect information related to the level of reporter gene expression. Reporter gene constructs are driven by upstream promoter/enhancer elements; reporter gene expression can be ''constitutive'' leading to continuous transcription and used to identify the site of transduction and to monitor the level and duration of gene (vector) activity. Alternatively, reporter gene expression can be ''inducible'' leading to controlled gene expression. Controlled gene expression can be tissue-specific and/or responsive to the level of endogenous promoters and transcription factors. Several examples of imaging endogenous biological processes in animals using reporter constructs, radiolabeled probes and PET imaging are reviewed, including: 1) imaging transcriptional regulation (e.g., p53-dependent gene expression), 2) imaging weak promoters (*cis*- vs. *trans*-reporter configurations), 3) imaging post-transcriptional regulation of gene expression, 4) imaging protein-protein interactions. The development of versatile and sensitive assays that do not require tissue sampling will be of considerable value for monitoring molecular-genetic and cellular processes in animal models of human disease, as well as for studies in human subjects in the future. Non-invasive imaging of molecular-genetic and cellular processes will compliment established ex vivo molecular-biological assays that require tissue sampling, and will provide a spatial as well as a temporal dimension to our understanding of various diseases. J. Cell. Biochem. Suppl. 39: 172–183, 2002.

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In vivo molecular-genetic imaging has its roots in molecular biology and cell biology, as well as in imaging technology. The molecular-genetic focus of three different non-invasive, in vivo imaging technologies developed more or less in parallel during the past decade: (1) magnetic resonance imaging [Weissleder et al., 1990, 1991, 1997; Kayyem et al., 1995; Louie et al., 2000]; (2) radionuclide imaging (gamma camera, PET, and quantitative aurtoradiography) [Tjuvajev et al., 1995, 1996, 1998; Gambhir et al., 1998, 1999]; and (3) optical imaging of small animals [Contag et al., 1997, 1998; Rehemtulla et al., 2000]. Imaging technology is rapidly evolving and during the past several years has begun to converge with the disciplines of molecular and cellular biology. This convergence provides a well-established foundation for developing new imaging research opportunities and for translation of these new imaging paradigms into clinical applications.

Molecular-genetic imaging provides visualization in space of normal as well as abnormal cellular processes at a molecular or genetic level rather than at the anatomical level. Needless

Abbreviations used: PET, positron emission tomography; FDG, fluorodeoxyglucose; HSV1-tk, HERPES simplex virus type 1 thymidine kinase; FIAU, 5-iodo-2′-fluoro-2′deoxy-1-β-D-arabino-furanosyl-uracil; FHBG, 9-(4-fluoro-3-hydroxymethylbutyl)guanine; ACV, acyclovir; GCV, ganciclovir; RASONs, radiolabeled small oligonucleotides; hD2R, human dopamine 2 receptor; hSSTR2, FESP, (fluoroethyl)-spiperone; human somatostatin receptor subtype-2; IRES, internal ribosome entry site; eGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site.

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to say, current gamma camera, PET, magnetic resonance, and optical technologies that are used to image animals and patients do not visualize individual cells, much less molecules. What is so exciting about this emerging new field relates to the novel imaging paradigms that are being developed. These paradigms can be successful within the inherent spatial resolution limits of existing imaging systems, because some degree of tissue (cell) homogeneity within the resolution elements (pixels) of the resultant images can be achieved. This brief review will primarily focus on radionuclide imaging, although many of the principles described are directly applicable to optical and magnetic resonance imaging technology as well. A more extensive discussion of these issues was recently published [Blasberg and Tjuvajev-Gelovani, 2002].

Established in vitro and in situ molecular assays require invasive sampling procedures that preclude sequential studies in the same animal or in human subjects. Tissue sampling may not always adequately represent the biochemical or pathological process under investigation due to tissue heterogeneity, which is especially characteristic of some tumors. Furthermore, temporal studies that employ molecular-biological assays often require large numbers of animals that are sacrificed at specific time points in order to achieve a statistically significant temporal profile. The development of sensitive imaging-based assays to monitor molecular-genetic and cellular processes in vivo and over time would be of considerable value in the study of animal models of human disease (including transgenic animals), as well as for studies in human subjects. Noninvasive imaging of molecular-genetic and cellular processes will compliment established ex vivo molecular-biological assays, and imaging can provide a spatial as well as a temporal dimension to our understanding of various diseases.

IMAGING STRATEGIES

Three imaging strategies—"direct," "indirect," and "surrogate"—are currently the most widely used. "Direct molecular imaging" can be defined in terms of a probe-target interaction, whereby the resultant image of probe localization and magnitude (image intensity) is directly related to its interaction with the target epitope or enzyme. This strategy is based on imaging the target directly, usually with a target-specific probe. "Indirect molecular imaging" is a little more complex in that it may involve multiple components. One example of indirect imaging that is now being widely used is "reporter imaging," which usually includes a "reporter gene" and a "reporter probe." The "reporter gene" product frequently is an enzyme that converts a "reporter probe" to a metabolite that is selectively trapped within transduced cells. Alternatively, the reporter gene product can be a receptor or transporter that "irreversibly traps" the probe in transduced cells. Indirect reporter imaging paradigms are currently more widely used in molecular imaging and will be discussed in greater detail below. "Surrogate" imaging strategies that reflect down-stream effects of one or more endogenous molecular/genetic processes are also being explored. This latter approach is particularly attractive for potential translation into clinical studies in the near-term, because radiopharmaceuticals and imaging paradigms that are already established in the clinic (or soon to be implemented) can be used in surrogate imaging. Surrogate imaging may be useful for monitoring down-stream effects of specific molecular-genetic pathways that are altered in diseases such as cancer. Examples of direct, indirect, and surrogate imaging using radiolabeled probes and PET to visualize endogenous molecular processes, such as the regulation of endogenous gene expression, will be discussed below.

"Direct" Imaging Strategies

"Direct" imaging strategies are used in all three imaging motifs. For example, monoclonal antibody or peptide-specific targeting of a particular cell membrane epitope can be imaged with a paramagnetic, florescent, or radionuclide-labeled probe. Imaging cell surface specific antigens or epitopes with radiolabeled antibodies is an example of direct molecular imaging that has developed over the past 30 years. Similarly, PET imaging of receptor density/occupancy using small radiolabeled molecular probes has also been widely used, particularly in neuroscience research, over the past two decades. These examples represent some of the first "molecular imaging" applications used in clinical nuclear medicine research. Other examples of direct imaging paradigms use

radiolabeled analogues of naturally occurring compounds, such as [^{18}F]-fluorodeoxyglucose (FDG) to image the glucose utilization in the brain which is based largely on the activity of a particular enzyme (hexokinase) and was described more than two decades ago [Reivich et al., 1977; Sokoloff et al., 1977], or imaging the activity of a particular transporter with a transport-specific probe [Blasberg et al., 1983; Miyagawa et al., 1998].

A more recent direct imaging strategy involves the development of antisense and aptomer oligonucleotide probes that specifically hybridize to target mRNA or proteins in vivo. Radiolabeled antisense probes are radiolabeled oligonucleotides (RASONs) that have been developed to directly image endogenous gene expression at the transcriptional level. RASON sequences can be made complimentary to a small segment of target mRNA or DNA, and could potentially target any specific mRNA or DNA sequence. In this context, imaging specific mRNAs with RASONs produces "direct" images of specific molecular-genetic events. Some efficacy for gamma camera and PET imaging endogenous gene expression using RASONs has been reported [Dewanjee et al., 1994; Tavitian et al., 1998]. Nevertheless, RASON imaging has several serious limitations, including: (a) low number of target mRNA/DNA molecules per cell; (b) limited delivery (poor cell membrane and vascular permeability, limited penetration of the blood-brain barrier); (c) poor stability (degradation by H-RNAse); (d) slow clearance (slow washout of non-bound oligonucleotides); (e) comparatively high background activity and low specificity of localization (low target/background ratios). Imaging specific RASON targets in the body is complicated and interpretation of the images must be approached with caution.

A constraint of direct imaging strategies is the necessity to develop a specific probe for each molecular target, and then to validate both sensitivity and specificity in the application of each newly developed probe. This can be very time consuming and costly (e.g., the development, validation, and regulatory approval for [^{18}F]-FDG PET imaging of glucose utilization in tumors has taken over 20 years), but it can also be very fruitful. This has been the traditional approach and reflects the considerable amount of time and effort required to develop a new probe to image a metabolic process in patients. Alternatively, it is now possible to develop and validate "indirect" imaging strategies more rapidly and at considerably lower cost using established reporter genes and reporter probes. Namely, a single or small number of reporter genes (in combination with a small number of reporter probes) can be used in many different reporter constructs to image many different processes. Indirect molecular imaging strategies are already providing the opportunity for a wider application of imaging in the study of experimental animal models of human disease, and for their implementation in future clinical studies.

"Indirect" Imaging Strategies

"Indirect" imaging strategies are currently most widely used for radionuclide-based molecular imaging [Tjuvajev et al., 1999; Yu et al., 2000] and for optical imaging [Mayerhofer et al., 1995; Sweeney et al., 1999; Rehemtulla et al., 2000], and to a lesser degree for MR imaging [Weissleder et al., 1997; Louie et al., 2000]. Most indirect molecular imaging paradigms involve the use of reporter-transgene technology which involves the coupling of a "reporter gene" with a complimentary "reporter probe." Imaging the level of reporter gene product activity through probe accumulation provides indirect information that reflects the level of reporter gene expression, and the level of endogenous signaling/transcription factors that drive reporter gene expression. Reporter gene imaging was initially developed and used with in situ optical technology that usually required post-mortem tissue sampling and processing (e.g., beta-galactosidase assay). More recent studies have emphasized non-invasive imaging techniques involving live animals (and soon in human subjects). Non-invasive reporter gene imaging involves a reporter transgene (e.g., HERPES simplex virus type 1 thymidine kinase gene, *HSV1-tk*) placed under the control of upstream promoter/enhancer elements. These promoter/enhancer elements can be "always turned on" with constitutive promoters (e.g., LTR, RSV, CMV), or they can be "sensitive" to activation by specific endogenous transcription factors (factors that bind to and activate specific promoter-enhancer elements). Several non-invasive imaging paradigms have been described and it has recently been shown that transcriptional regulation of endogenous (host tissue) gene expression can be imaged using both nuclear (PET) and optical (fluorescence) imaging [Doubrovin et al.,

2001; Ponomarev et al., 2001]. Combined PET and bioluminescence imaging reporters have also been developed [Iyer et al., 2001], and combined triple-reporter (PET, fluorescence, and bioluminescence) imaging has also been shown to be effective and very useful [Ponomarev et al., unpublished results].

Reporter Gene Imaging

A common feature of all reporter vectors is the cDNA expression cassette containing the reporter transgene(s) of interest (e.g., *HSV1-tk*). The advantage and versatility of reporter vectors is that the design and arrangement of the expression cassette can be varied. For example, the reporter transgene(s) can be driven by any promoter/enhancer sequence of choice. The promoter can be "constitutive" (leading to continuous transcription), or it can be "inducible" (leading to controlled expression). The promoter can also be cell specific, allowing expression of the transgene to be restricted to certain cells and organs. The paradigm for quantitative imaging of transgene expression involves several steps, including the initiation of transcription (that can be controlled by specific promoter/enhancer elements), the process of DNA transcription and stabilization of mRNA, and subsequent translation of mRNA into the gene product (a protein). In this manner the reporter expression cassette can be designed to provide information about endogenous gene regulation, mRNA stabilization, and specific protein–protein interactions.

A general paradigm for gene imaging using radiolabeled probes was initially described in 1995 [Tjuvajev et al., 1995] and is diagrammatically shown in Figure 1. This paradigm requires the selection of an appropriate combination of reporter/marker transgene and reporter/marker probe. It is important to note that imaging transgene expression is independent of the vector used to transfect/transduce target tissue; namely, any of several currently available vectors can be used (e.g., retrovirus, adenovirus, adeno-associated virus, lentivirus, liposomes, etc.). The reporter transgene can encode for an enzyme, (e.g., *HSV1-tk*), a receptor (e.g., *hD2R* and *hSSTR2*), or a transporter (e.g., *hNIS*).

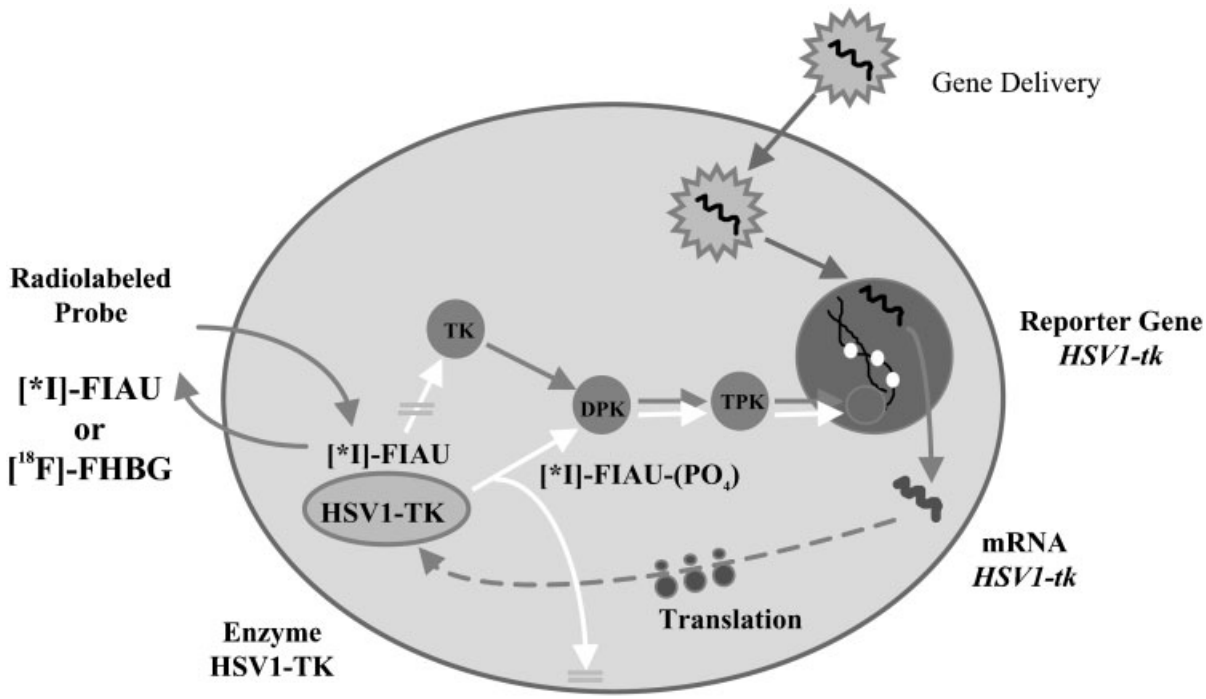

Fig. 1. Schematic for imaging HSV1-tk reporter gene expression with reporter probes FIAU and FHBG. The *HSV1-tk* gene complex is transfected into target cells by a vector. Inside the transfected cell, the *HSV1-tk* gene is transcribed to HSV1-tk mRNA and then translated on the ribosomes to a protein (enzyme), HSV1-TK. After administration of a radiolabeled probe and its transport into the cell, the probe is phosphorylated by HSV1-TK (gene product). The phosphorylated radiolabeled probe does not readily cross the cell membrane and is ''trapped'' within the cell. Thus, the magnitude of probe accumulation in the cell (level of radioactivity) reflects the level of HSV1-TK enzyme activity and level of HSV1-tk gene expression.

Wild-type *HSV1-tk* [Tjuvajev et al., 1996] or a mutant *HSV1-tk* gene, *HSV1-sr39tk* [Gambhir et al., 2000], are the reporter genes most commonly used in current molecular imaging studies using radiolabeled probes and PET imaging. The *HSV1-tk* and *HSV1-sr39tk* gene products are proteins (enzymes) that have less substrate specificity than mammalian thymidine kinase 1 (TK1). The viral kinases phosphorylate a wider range of compounds, including acycloguanosines (e.g., acyclovir, ACV; ganciclovir, GCV; 9-[4-fluoro-3-(hydoxymethyl)butyl]guanine, FHBG) and 2′-fluoronucleoside analogues of thymidine (e.g., 5-iodo-2′-fluoro-2′deoxy-1-β-D-arabino-furanosyl-uracil, FIAU). This difference between mammalian and viral TK enzymes permits the development and use of radiolabeled probes that are phosphorylated to a significantly greater extent by HSV1-TK or HSV1-sr39TK in comparison to mammalian TK1. These viral gene products selectively metabolize a complimentary radiolabeled probe that results in its entrapment and accumulation in the transduced cell. A paired-comparison of different radiolabeled probes for imaging *HSV1-tk* expression has been reported recently [Tjuvajev et al., 2002].

It may be helpful to consider this reporter imaging paradigm as an example of an in vivo enzymatic radiotracer assay that reflects reporter gene expression. Enzymatic amplification of the signal (e.g., level of radioactivity) facilitates imaging the location and magnitude of reporter gene expression. Viewed from this perspective, reporter gene imaging using *HSV1-tk* is similar to imaging hexokinase activity with fluorodeoxyglucose (FDG).

A reporter gene can also encode for an extracellular or intracellular receptor or a cell membrane transporter (e.g., hD2R, hSSTR2, or hNIS) that binds or transports a radiolabeled or paramagnetic probe. The human dopamine 2 receptor (hD2R) [MacLaren et al., 1999], the human somatostatin receptor subtype-2 (hSSTR2) [Rogers et al., 2000], and the human sodium iodide symporter (*hNIS*) [Haberkorn, 2001] genes have been suggested as potential reporter genes for human studies. All three human genes have limited expression in the body; hD2R expression is limited to the striatal-nigral system of the brain, high hSSTR2 expression is largely limited to carcinoid tumors, and hNIS expression is limited largely to thyroid, stomach, and salivary glands. This approach is a very clever strategy because the reporter gene products are less likely to be immunogenic and there are established complimentary radiolabeled probes for each of these reporter genes that are approved for human administration: 3-(2′-[^{18}F]fluoroethyl)spiperone (FESP) for hD2R imaging [Barrio et al., 1989], [^{111}In]DTPA-octreotide (a complimentary radiolabeled somatostatin analogue) for hSSTR2 imaging [Hemminki et al., 2001], and radiolabeled iodide or pertechnetate for *hNIS* imaging [Haberkorn, 2001; Moon et al., 2001]. These three reporter systems have distinct benefits and are good candidates with respect to regulatory approval for molecular/reporter imaging in human subjects. However, receptor and transporter expression on the surface of cells is a complex process and involves intracellular trafficking and cell membrane incorporation that is likely to be altered under different conditions and different disease states. It remains to be shown whether imaging receptor and transporter-based reporters (e.g., the *hD2R*, *hSSTR2*, and *hNIS* systems) will provide a consistent and reliable measure of reporter gene expression under variable stress or altered conditions. In any case, the level of probe accumulation (level of radioactivity) must be shown to be proportional to the level of gene expression.

Surrogate Imaging

"Surrogate marker" probes that reflect the down-stream effects of one or more endogenous molecular/genetic processes are also being explored. This latter approach is particularly attractive for potential translation into clinical studies in the near-term, since direct and indirect molecular imaging studies are only beginning to be implemented in the clinic [Jacobs et al., 2001]. Existing radiopharmaceuticals and imaging paradigms may be useful for monitoring down-stream effects of changes in specific molecular/genetic pathways in diseases such as cancer. The "surrogate marker" approach is very likely to be less specific and more limited with respect to the number of molecular-genetic processes that can be imaged. Nevertheless, it benefits from the use of probes that have already been developed and studied in human subjects. Thus, the translation of "surrogate marker" imaging paradigms into patients will be far easier than either the

reporter transgene and direct imaging paradigms outlined above.

It remains to be shown whether there is a sufficiently high correlation between "surrogate marker" imaging and direct molecular assays that reflects the activity of a particular molecular/genetic pathway of interest. Very few studies have attempted a rigorous correlation between "surrogate marker" imaging and transcriptional activity of a particular gene, or post-transcriptional processing of the gene product, or the activity of a specific signal transduction pathway that is targeted by a particular drug. The application of "surrogate marker" imaging for monitoring treatment response is gaining increasing attention, particularly as it relates to the development and testing of new pathway-specific drugs. For example, the assessment of non-cytotoxic, cytostatic drugs, such as the anti-angiogenic class of drugs, pose particular problems for imaging assessments based on tumor volume. Thus, surrogate imaging markers of tumor vascularity are being proposed for assessing anti-angiogenesis treatment response. Whether imaging "surrogate markers" will be of value for assessing treatment directed at other molecular/genetic abnormalities in tumors (EGFR, p53, c-Met, HIF-1, etc) remains to be demonstrated.

IMAGING ENDOGENOUS BIOLOGICAL PROCESSES

Several indirect imaging strategies using reporter-gene technology have been used to visualize transcriptional and post-transcriptional regulation of target gene expression, as well as specific intracellular protein–protein interactions. Several examples are provided below.

Imaging Transcriptional Regulation

Imaging transcriptional regulation of endogenous genes in living animals (and potentially in human subjects) using non-invasive imaging techniques can provide a better understanding of normal and cancer-related biological processes. A recent study from our group was the first to show that p53 dependent gene expression can be imaged in vivo with PET and by in situ fluorescence [Doubrovin et al., 2001]. A retroviral vector (*Cis*-p53/*TKeGFP*) was generated by placing the HERPES simplex virus type 1 thymidine kinase (*tk*) and enhanced green fluorescent protein (e*gfp*) fusion gene (*TKeGFP*, a dual-reporter gene) under control of a p53-specific response element. DNA damage-induced upregulation of p53 transcriptional activity was demonstrated and correlated with the expression of p53-dependent downstream genes (including p21). These findings were observed in U87 (p53 +/+) cells and xenografts, but not in SaOS (p53 −/−) cells. This was the first demonstration that a Cis-reporter system (Cis-p53/TKGFP) was sufficiently sensitive to image endogenous gene expression using non-invasive nuclear (PET) imaging (Fig. 2A). The PET images corresponded with up-regulation of genes in the p53 signal transduction pathway (p53-dependent genes) in response to DNA damage induced by BCNU chemotherapy (Figs. 2B,C). PET imaging of p53 transcriptional activity in tumors using the Cis-p53TKGFP reporter system could be used to assess the effects of new drugs or other novel therapeutic paradigms that are mediated through p53-dependent pathways. For example, specific p53 gene therapy strategies that are based on p53 over expression [Merritt et al., 2001] could be monitored by non-invasive imaging.

It should also be pointed out that the dual reporter construct (*TKeGFP*-fusion gene) provides the opportunity for multi-modality (both nuclear and optical imaging) imaging of endogenous gene expression in vivo. The *TKeGFP* reporter gene could be introduced into other reporter assay systems to assess other molecular-biological pathways. It should also be possible to use the *TKeGFP* reporter gene in transgenic animals; this will facilitate the monitoring and assessment of newly cloned genes or novel signal transduction pathways. Another advantage of the dual reporter system is the ability to compare the images of reporter gene expression obtained with PET, gamma camera, or autoradiography with corresponding in situ GFP fluorescence images. The comparison between GFP fluorescence and autoradiographic images, coupled with histology of corresponding tissue sections provides for spatial and quantitative assessments of reporter gene expression at the microscopic as well as macroscopic level.

Imaging Weak Promoters

Imaging weak promoters is hampered when poor transcriptional activity of the reporter gene occurs when the promoter is in it usual

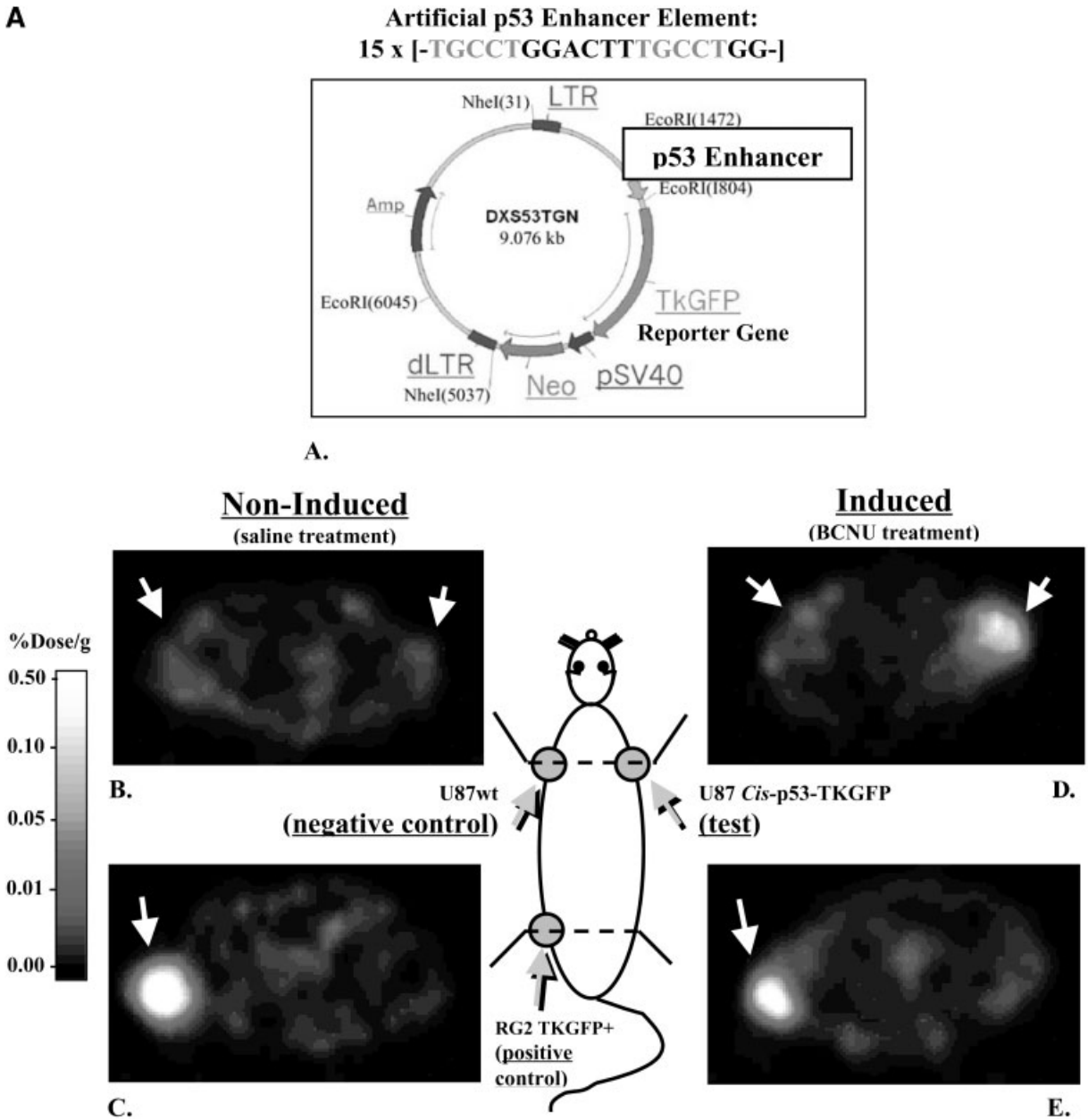

Fig. 2. PET imaging of endogenous p53 activation and validation of Cis-p53/TKGFP reporter system in cell cultures and sampled tumor tissue. The p53-sensitive reporter vector (**A**) contains an artificial p53 specific enhancer that activates expression of the *TKeGFP* reporter gene. Transaxial PET images (GE Advance tomograph) through the shoulder (**B**, **D**) and pelvis (**C**, **E**) of two rats are shown (**upper panel**); the images are color-coded to the same radioactivity scale (%dose/g). An untreated animal is shown on the left, and a BCNU-treated animal is shown on the right. Both animals have three s.c. tumor xenografts: U87p53TKGFP (test) in the right shoulder, U87 wild-type (negative control) in the left shoulder, and RG2TKGFP (positive control) in the left thigh. The non-treated animal on the left shows localization of radioactivity only in the positive control tumor (RG2TKGFP); the test (U87p53TKGFP), and negative control (U87wt) tumors are at background levels. The BCNU-treated animal on the right shows significant radioactivity localization in the test tumor (right shoulder) and in the positive control (left thigh), but no radioactivity above background in the negative control (left shoulder). Fluorescence microscopy and FACS analysis of a transduced U87p53/TKGFP cell population in the non-induced (control) state (**F**, **H**), and 24 h after a 2 h treatment with BCNU 40 μg/ml (**G**, **I**). Fluorescence microscopic images of U87p53/TKGFP s.c. tumor samples obtained from non-treated rats (**J**) and rats treated with 40 mg/kg BCNU i.p. (**K**). The RT-PCR blots from in vitro (**L**) and in vivo (**M**) experiments show very low HSV1-*tk* expression in non-treated U87p53TKGFP transduced cells and xenografts-bearing animals, respectively, and no HSV1-*tk* expression in wild-type U87 cells and tumor tissue, respectively. When U87p53TKGFP transduced cells and xenografts-bearing animals are treated with BCNU, there is a marked increase in HSV1-*tk* expression comparable to that in constitutively HSV1-*tk* expressing RG2TK + cells and xenografts. Figure adapted from Doubrovin et al. [2001].

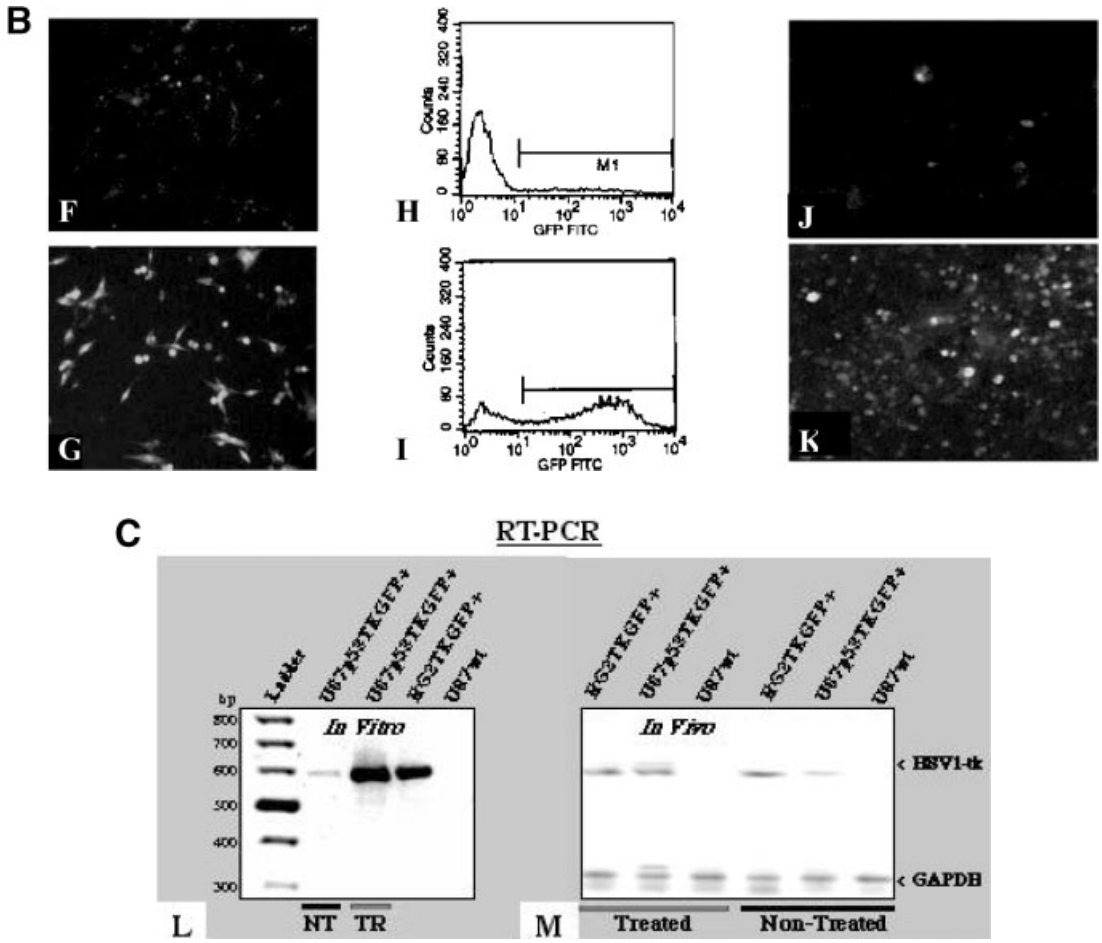

Fig. 2. (*Continued*)

Cis-configuration (e.g., CEA, PSE, etc.). In one study, [Qiao et al., 2002] validated methods to enhance the transcriptional activity of the carcinoembryonic antigen (CEA) promoter using a trans- or so called "two-step transcriptional amplification" (TSTA) system. To increase promoter strength while maintaining tissue specificity, a recombinant adenovirus was constructed which contained a TSTA system with a tumor-specific CEA promoter driving a transcription transactivator, which then activates a minimal promoter to drive expression of the *HSV1-tk* suicide/reporter gene. This ADV/CEA-binary-tk system resulted in equal or greater cell killing of transduced cells by ganciclovir in a CEA-specific manner, compared with ganciclovir killing of cells transduced with a CEA-independent vector containing a constitutive viral promoter driving HSV-tk expression (ADV/RSV-tk). In another study, [Iyer et al., 2001] validated methods to enhance the transcriptional activity of the androgen-responsive prostate-specific antigen promoter (PSA) using a similar TSTA approach to amplify expression of firefly luciferase and mutant HERPES simplex virus type 1 thymidine kinase (HSV1-sr39tk) in a prostate cancer cell line (LNCaP). Further improvements of the androgen-responsive TSTA system for reporter gene expression were made using a "chimeric" TSTA system that uses duplicated variants of the prostate-specific antigen (PSA) gene enhancer to express GAL4 derivatives fused to one, two, or four VP16 activation domains. A very encouraging result was the demonstration that the TSTA system was androgen concentration sensitive, suggesting a continuous rather than binary reporter response. However, as observed with the CEA-TSTA reporter system above, the in vivo imaging comparison of the TSTA and

Cis-reporter systems showed substantially less dramatic differences than that obtained by the in vitro analyses.

Imaging Post-Transcriptional Regulation of Gene Expression

Imaging post-transcriptional regulation of gene expression is a new observation and is based on the following paradigm. Human cells exposed to antifolates show a rapid increase in the levels of the enzyme dihydrofolate reductase (DHFR). Several studies indicated that DHFR enzyme binds to it's own mRNA in the coding region, and that inhibition of DHFR by MTX releases the DHFR enzyme from the mRNA. Consequently, this release results in an increase in translation to DHFR protein [Ercikan-Abali et al., 1993]. In addition to the described translational regulation of DHFR in cancer cells exposed to MTX, increased levels of DHFR also result through DHFR gene amplification, a common mechanism of acquired resistance to this drug. In contrast to rapid translational modulation of DHFR, gene amplification occurs in response to chronic exposure to antifolates, and elevated cellular levels of DHFR result from transcription of multiple DHFR gene copies. Recently, [Mayer-Kuckuk et al., 2002], showed that this adaptive cellular response mechanism could be used to determine whether post-transcriptional regulation of gene expression could be monitored by reporter-PET imaging. The results of this study indicated that the increase in reporter protein and enzyme (DHFR-HSV1TK) activity was occurring at a translational level, rather than at the transcriptional level. This effect could be visualized by [^{124}I]FIAU and PET imaging studies that were performed on nude rats bearing DHFR-HSV1TK-transduced HCT-8 xenografts, demonstrating a proof of principle.

Imaging Protein–Protein Interactions

Imaging protein–protein interactions was also demonstrated recently in vivo by two studies [Luker et al., 2002; Ray et al., 2002]. The interaction of two proteins, ID and MyoD, was the focus of the first study. To modulate the expression of these two proteins, the NF-κB promoter was used to drive expression of the *id-gal4* and/or *myoD-vp16* fusion genes, and tumor necrosis factor (TNF-α) was used to induce the NF-κB promoter. The reporter construct contained five GAL4 DNA-binding sites driving the expression of the firefly luciferase (*fl*) reporter gene. Eight hours after TNF-α administration, the mice showed a significantly greater level of *fl* expression (~20-fold at 8 h; fivefold at 20 h; and threefold at 30 h) when compared with mice that did not receive TNF-α; and ~60-fold greater than that in mice injected with 293T cells transfected with non-interacting protein partners (MyoD and p53).

In the second study, protein–protein interactions could be imaged in vivo with PET and by fluorescence imaging using a variant of HSV1-tk/GFP dual reporter fusion gene [Luker et al., 2002]. It is well known that TAg, which is the transforming protein of SV40 virus, binds constitutively to p53 and blocks transactivation of p53 target genes, leading to transformation of mammalian cells. In this study, the authors used an approach similar to that described previously by [Ray et al., 2002], but they used a somewhat different reporter system involving a reverse tetracycline-responsive transactivator expressed from a cytomegalovirus promoter and a plasmid expressing either Gal4-BD-p53 and VP16-TAg or Gal4-BD-p53 and VP16-CP from a bidirectional, tetracycline-regulated promoter. Mice bearing the test and control transduced xenografts were treated with doxycycline for 48 h to induce expression of the hybrid proteins. Visualization of the Gal4-mNLS-sr39TK-EGFP reporter at 48 h was accomplished with [^{18}F]-FHBG and microPET imaging. Based on region-of-interest values from the micro-PET images, the uptake of ^{18}F-FHBG was 5.5-fold greater in TAg (test) xenografts compared to that in CP (control) xenografts.

These initial results are very encouraging, but at the same time they are also limited because they demonstrate only constitutive interactions of the known pairs of interacting proteins. Further studies will be required to validate the approach with respect to the sensitivity and dynamic range of these reporter systems for monitoring the induction and inhibition of endogenous protein–protein interactions. Namely, these approaches cannot fully identify the temporal kinetics of gene expression or protein–protein interaction. If target gene expression or a protein–protein interaction will change (e.g., decrease or stop) as the result of a change in signal transduction activity, this change may not be readily detected due to persistence of the already expressed

reporter protein (determined by the half-life of the reporter protein). Also, following protein–protein interactions, these reporter systems may require significantly longer periods of time to reach sufficient levels reporter-protein expression because adequate levels of both chimeric interactive proteins must be achieved before transactivation of the reporter gene can occur. Future studies are necessary to look at the half-lives of the fusion proteins, reporter mRNA, and different reporter proteins (e.g., destabilized short-lived reporters) will help to optimize the kinetics of different reporter systems.

ISSUES FOR THE FUTURE

The opportunities for molecular imaging (and biomedical imaging as a whole) indeed look bright. Non-invasive reporter gene imaging is a very exciting indirect imaging strategy that can be fully exploited in experimental and transgenic animals. However, reporter gene imaging applications will be more limited in patients due to the necessity of transducing target tissue with specific reporter constructs. Ideal vectors for targeting specific organs or tissue (tumors) in patients do not exist at this time, although vector development is a very active area of human gene therapy research. Each new imaging probe and each new vector requires extensive and time-consuming safety testing prior to government approval for human administration. However, once a reporter-gene and reporter-probe combination has been validated and approved for human studies, this reporter system can potentially be used in almost any vector. That is not the case with direct imaging probes, where the number of probes is closely related to the number of potential direct imaging targets. Although highly specific images can be obtained with direct imaging probes, the development and validation of new probes is similar to the development, testing and validation of new drugs. In contrast, the development of different reporter gene constructs and their validation is far simpler than that for new imaging probes. The wider application and more rapid development of reporter-imaging systems is likely to result in the translation of reporter gene imaging into patient studies sooner than the application of corresponding direct imaging probes. Direct imaging probes do have specific advantages and they will continue to be developed and make significant contributions to molecular imaging, although at a slower pace. A major advantage of direct imaging probes is that once developed and validated, they do not require the transduction of target tissue by a reporter gene-bearing vector.

Government approval will be required for all new vectors and all new direct imaging probes prior to their human administration. The translation of molecular imaging research into patient studies and clinical application must proceed step-wise and must be carefully monitored. In this context there may be a place for "surrogate" imaging in the near future; this will depend on demonstrating a reasonable correlation between the surrogate image/measure and direct molecular assays that define the activity of the particular molecular/genetic pathway of interest.

The benefits of non-invasive monitoring (imaging) of transgene expression in gene therapy protocols are substantial; it will provide a practical and clinically useful way to identify successful gene transduction and expression in target (and non-target) tissue over time. The ability to visualize transcriptional and post-transcriptional regulation of endogenous target gene expression, as well as specific intracellular protein–protein interactions in patients will provide the opportunity for new experimental venues for research in patients. For example, it may be possible to image a drug's effect on a specific regulatory or signal transduction pathway in an individual patient's tumor. The development of versatile and sensitive assays that do not require tissue samples will be of considerable value for monitoring molecular-genetic and cellular processes in animal models of human disease, as well as for studies in human subjects in the future. Non-invasive imaging of molecular-genetic and cellular processes will compliment established ex vivo molecular-biological assays that require tissue sampling, and imaging will provide a spatial as well as a temporal dimension to our understanding of various diseases.

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