Optical Bioluminescence and Positron Emission Tomography Imaging of a Novel Fusion Reporter Gene in Tumor Xenografts of Living Mice

Pritha Ray, Anna M. Wu, and Sanjiv S. Gambhir

Abstract

Noninvasive imaging of reporter gene expression using various imaging modalities is playing an increasingly important role in defining molecular events in the field of cancer biology, cell biology, and gene therapy. In this study, a novel reporter vector was constructed encoding a fusion protein comprised of a mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk) (tk), a positron emission tomography (PET) reporter gene, and renilla luciferase (rl), a bioluminescence optical reporter gene joined by a 20 amino acid long spacer sequence. We validated the activity of the two enzymes encoded by the fusion protein (tk-rl) in cell culture. Then, tumors stably expressing the tk-rl fusion gene were imaged both by microPET and optically using a cooled charge coupled device camera in xenograft-bearing living mice. Using a single fusion reporter (PET/optical) gene should accelerate the validation of reporter gene approaches developed in cell culture for translation into preclinical and clinical models.

Introduction

It is important to be able to image reporter gene expression in living cells, animals, and humans using a single reporter construct. A single reporter gene would facilitate rapid translation of approaches developed in cells to preclinical models and clinical applications. To date, various methodologies exist that allow the imaging of reporter gene expression in living cells and animals noninvasively and repetitively. Confocal laser microscopy, two-photon laser microscopy, and several other techniques are available for real-time imaging of gene expression at the single cell level using fluorescence (1, 2). For reporter gene imaging in living subjects PET, single photon emission computed tomography, magnetic resonance imaging, and optical imaging are well standardized and are being used extensively in small animal models (3–5) and more recently with PET in humans (6, 7). These imaging techniques play important roles in defining critical pathways involved in tumorigenesis, metastasis, and evaluating the efficiency of gene therapy strategies (8–10). Each of these modalities has unique applications, advantages, and limitations that can be complementary to other modalities. A cell-based technique is not useful for whole body in vivo imaging studies, whereas techniques involved in imaging at the tissue or organism level do not have the resolution power to image gene expression at the cellular level. Among the whole body imaging modalities, the radionuclide-based techniques have high sensitivity, good spatial resolution, and are tomographic in nature but are somewhat limited by their higher cost and especially for PET, the need for a cyclotron for production of isotopes for most tracers. In contrast, optical imaging techniques (fluorescence and bioluminescence) represent a low cost and quick alternative for real-time analysis of gene expression in small animal models (4, 10) but are limited by depth penetration and cannot be easily generalized to human applications.

To overcome the shortcomings of each modality, a multimodality approach should be very useful for detecting reporter gene expression. Combining two different technologies (e.g., PET with optical) through a unified vector would have the advantage of speed and ease of validating approaches in small animals that in turn can be translated to humans. Such a vector might be achieved by several different approaches. A single reporter gene can be investigated for a single substrate doubly labeled with different signatures such as a radioactive nuclide (suitable for radionuclide imaging) or a nonradioactive paramagnetic/bioluminescent/fluorescent molecule (suitable for magnetic resonance or optical imaging) and thus can be imaged by different imaging modalities. However, development of such substrates is often difficult because of the complex chemical nature of the biomolecules and limitations on required pharmacokinetics in vivo. On the other hand, a single vector can be designed harboring two different reporter genes imaged by two different techniques (e.g., one radionuclide and one optical; Fig. 1). Coexpression of two genes is generally achieved by using dual promoters, by insertion of an internal ribosomal entry site or by fusing the two genes into a single translational cassette (3). Our laboratory has successfully used tk (HSV1-sr39 thymidine kinase, an improved PET reporter gene over the wild-type HSV1-tk when using the guanosine analogues as tracer) and rl (renilla luciferase, a bioluminescence optical reporter gene) as separate imaging tools for studying the location, magnitude, and time variation of reporter gene expression in living subjects (5, 11, 12). In this study, we report the construction and validation of a novel tk and rl fusion protein imaged by microPET and bioluminescent optical CCD imaging modalities in tumor xenograft-bearing living mice.

Materials and Methods

Chemicals. FDG was synthesized at University of California at Los Angeles as described previously (13). 8-1H-Penciclovir was obtained from Moravek Biochemicals (Brea, CA). FHBG (9-4-[18F]fluoro-3-hydroxymethylbutylguanine) and [CANCER RESEARCH 63, 1160 –1165, March 15, 2003] FDG was synthesized at University of California at Los Angeles as described previously (13). 8-1H-Penciclovir was obtained from Moravek Biochemicals (Brea, CA). FHBG (9-4-[18F]fluoro-3-hydroxymethylbutylguanine) and
MULTIMODALITY IMAGING OF A FUSION REPORTER GENE

Fig. 1. Measuring reporter gene expression using two different imaging modalities when two reporter genes are simultaneously expressed from a fusion vector. tk, a PET reporter gene is fused with rl (renilla luciferase), an optical bioluminescence reporter gene with the help of a polynucleotide coding for a short 20 a.a. long spacer and expression is driven by a CMV promoter. Transcription of this fusion vector yields a single mRNA, and subsequent translation leads to a single polypeptide that is capable of retaining partial if not full activities of the two proteins fused. Noninvasive, quantitative, and repeated imaging of the location and magnitude of the both reporter gene expression either by trapping of a PET reporter probe (e.g., FHBG phosphorylated by TK enzyme) or by catalysis of an optical reporter probe (e.g., production of light through RL-coelenterazine reaction) can be imaged by both microPET and optical CCD camera in living subjects, respectively. (Biosignal Packard, Montreal, Quebec, Canada). For PCR amplification, three different 3′ end primers (5′-GAGCCGATGCTTGGCCTCCCCCAT-3′; 5′-GAGCCGATGCTTGGCCTCCCCCAT-3′; 5′-GAGCCGATGCTTGGCCTCCCCCAT-3′) were used along with the same 5′ end primer (5′-CGAGCTAGCCGACCTGCTTACCACCTC3′) to eliminate the stop codon of the tk gene and introduce different restriction sites. Cloning of these three different PCR products of tk gene into three subtypes of R-Luc N fusion vector (N1, N2, and N3) that differ from each other by 1 or 2 bases in their multicloning sites to provide alternate reading frames) generated spacers differing in length, sequence, and composition. Cloning of these three different PCR products of tk gene into three subtypes of R-Luc N fusion vector (N1, N2, and N3) that differ from each other by 1 or 2 bases in their multicloning sites to provide alternate reading frames) generated spacers differing in length, sequence, and composition. Cloning of these three different PCR products of tk gene into three subtypes of R-Luc N fusion vector (N1, N2, and N3) that differ from each other by 1 or 2 bases in their multicloning sites to provide alternate reading frames) generated spacers differing in length, sequence, and composition.

Cell Lines, Transfection Procedures, and Stable Clone Isolation. C6 rat glioma cells (obtained from Dr. M. Black), N2a neuronal cell lines (obtained from Dr. Vincent Mauro, Scripps Research Institute, La Jolla, CA), and 293T human embryonic kidney cells (American Type Culture Collection, Manassas, VA) were used. The C6 cells were cultured in high glucose, deficient minimal Eagle’s medium supplemented with 5% FBS and 1% penicillin (100 µg/ml), streptomycin (292 µg/ml), glutamine (100 mM), and histidinol (27 µg/ml) by volume. The N2a cells were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin (100 µg/ml), streptomycin (292 µg/ml), and 293T cells were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. All transient and stable transfections were carried out using the Qiagen Superfect transfection reagent (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer. The N2a stable cell lines carrying the fusion gene construct were selected with 200 µg/ml of G418. The clones were initially screened for renilla luciferase activity using a CCD camera (5) and then assayed for thymidine kinase activity (14).

TK, RL, and β-Gal Activity. Thymidine kinase activity assays were performed as previously described (14), and β-gal and renilla luciferase assays were done using the β-Gal enzyme assay system and Dual-Luciferase Reporter Assay System from Promega (Madison, WI), respectively.

Western Blot Analysis. The expression of TK and RL were evaluated by Western blotting with a rabbit polyclonal anti-TK antiserum and a mouse monoclonal antirenilla antibody using cell lysates prepared from 293T cells transfected with tk-rl, HSV1-sr39tk, or rl plasmids (11). A semiquantitative analysis of the Western blot was performed using the MacBAS V2.4 software (Fuji Base 5000, Tokyo, Japan).

MicroPET Imaging of Mice. Animal care and euthanasia were performed with the approval of the University of California Animal Research Committee. Twelve- to 14-week old male nude mice (nu/nu) were injected s.c. with −2 × 108 of N2a cells stably expressing the tk-rl fusion and control nonexpressing N2a cells, and after 8–10 days, tumor-bearing mice were scanned in microPET as described earlier (11). The microPET images were reconstructed by using three-dimensional filtered back projection and an iterative maximum a posteriori algorithm (15). ROIs were drawn over the tumor area. The ROI counts were converted to the %ID/g tumor using filtered back projection as previously described (11), and images shown were reconstructed with maximum a posteriori algorithm.

Optical Imaging of Renilla Luciferase Expression in Vivo. For in vivo optical imaging, mice implanted with stably expressing tk-rl fusion N2a, and control N2a cells were anesthetized and each mouse was then injected with 10 µl of coelenterazine (stock solution, 2 µg/µl in methanol) diluted in 90 µl of PBS (pH 7) via tail vein. Each animal was then placed supine in a light tight chamber, and whole body images were obtained and quantified as described previously (5).

Results

A HSV1-sr39 Thymidine Kinase PET and Renilla Luciferase Bioluminescence Reporter Gene Fusion Vector Bearing the Coding Sequence for a 20 a.a. Long Spacer Maintains the Highest TK and RL Activity in Three Different Cell Lines. We first constructed a fusion gene vector carrying tk and rl reporter genes using three spacer lengths. The PCR-amplified tk gene fragments from the pCDNA3.1-HSV1-sr39tk plasmid (12) were cloned in frame into R-Luc-N fusion vectors to generate a fusion gene construct under the CMV promoter. PCR amplification of tk gene using three different 3′ end primers and subsequent cloning of these amplified fragments generated three tk-rl fusion constructs bearing spacers with length and sequence as indicated: tk20rl (NHSVAGYQACGTAGPGSTG); tk18rl (SRVCRISLLRYRPRTG); and tk10rl (AVPRARDPTG). Plasmid DNA prepared from four to five clones for each spacer type were transiently transfected in 293T cells and assayed for TK and RL activity. The plasmid clones exhibiting the highest TK and RL activities were selected for additional studies (data not shown). Each of the three tk-rl fusion constructs were then subsequently cloned in pCDNA3.1 (+) backbone to directly compare results of each fusion with the pCDNA3.1-HSV1-sr39tk and pCDNA3.1-rl [rl was also cloned in pCDNA3.1 (+) from the R-Luc-N fusion vector], the positive control plasmids.

To compare the levels of reporter gene expression of each tk-rl fusion plasmids, three different cell lines [293T (Fig. 2A.1), N2a (Fig. 2A.2), and C6 (data not shown)] were transiently transfected with the three plasmids (tk20rl, tk18rl, or tk10rl) along with positive controls (pCDNA 3.1-rl and pCDNA3.1-rl) and negative controls (control cells mock transfected). Each cell line was also cotransfected with the β-gal reporter gene to normalize for transfection efficiency. After 24 h, the expression levels of all of the three reporter genes were assayed from the same cell lysates and TK and RL activities were normalized to β-GAL activity. Despite decreased TK enzyme activity seen by all of the fusion constructs in comparison to the positive control (pCDNA3.1-HSV1-sr39tk), a trend of increase in the level of TK activity with increasing spacer length is observed. The tk20rl plasmid (longest spacer) shows the highest TK activity, which is still 45% (293T; Fig. 2A.1), 19% (N2a; Fig. 2A.2), or 22% (C6; data not shown) of that of the positive control. Interestingly, the RL activity of each construct is relatively higher (~6–8 fold; Fig. 2A.1 and A.2) than the positive control (pCDNA3.1-rl) and also increases with increasing spacer length.
Western Blot Analysis of Extracts from Cells Transiently Transfected with tk-rl Probed with anti-TK and anti-RL Antibodies Reveals the Presence of an 80-kDa Fragment, the Predicted Size of the TK-RL Fusion. To investigate tk-rl fusion reporter gene expression at the protein level, cell lysates from tk-rl-, tk-, and rl-transfected 293T cells were resolved by 10% SDS-PAGE and analyzed on Western blots by using antibodies specific for TK or RL. The predominant band recognized by both anti-TK and anti-RL antibodies is of 80 kDa, the expected size of the TK-RL fusion protein. This is likely attributable to partial cleavage of the fusion protein. TK and RL proteins were recognized at ~46 kDa and 36 kDa band by their specific antibodies. A semiquantitative analysis of the Western blot revealed that ~36 and 25% of the total fusion protein was cleaved into its TK and RL components, respectively. The lower molecular weight bands present in the positive TK sample might have resulted from partial degradation of the sample or nonspecific binding of the polyclonal anti-TK antibody to other cellular proteins.

N2a Cells Stably Expressing the tk-rl Fusion Reporter Gene Can Be Imagined in Living Mice Using a Tumor Xenograft Model by both microPET and Optical-cooled CCD Imaging Systems. Our aim of building a fusion reporter vector was to test its efficacy for imaging of reporter gene expression quantitatively and repeatedly in living subjects using different modalities. We therefore isolated several clones of N2a cells stably expressing tk-rl fusion gene and one exhibiting the highest TK, and RL activity was tested for its ability to be imaged in vivo using microPET and a cooled CCD camera in a tumor xenograft model. Five nude mice received s.c. injections in each shoulder with control N2a cells or tk-rl-expressing cells. When the tumors attained a minimum of 0.6 cm in diameter, mice were first scanned using the cooled CCD camera followed by a microPET scan. Optical imaging of these mice after tail-vein injection of coelenterazine reveal that the tumors expressing the tk-rl fusion show relatively high bioluminescence of ~3081 × 10^3 + 725 × 10^3 maximum (p/sec/cm^2/sr) in comparison to the control N2a tumors (~3.1 × 10^3 + 0.7 × 10^3 maximum (p/sec/cm^2/sr), P < 0.002; Fig. 3A). Next, we scanned these mice by microPET using FHBG and finally on the following day using FDG. We quantified the signal from each tumor directly from the microPET images to determine the %ID/g for FDG and FHBG. This %ID/g is a measure of the amount of tracer accumulated in a given tissue site normalized to the injected amount and to the mass of the tissue examined. The FHBG accumulation in the tumors reflects the TK activity of the tk-rl-expressing cells, whereas the FDG accumulation reflects the metabolic activity of the tumor cells. The mean %ID/g value for FHBG accumulation in the tk-rl-expressing tumors (0.812 ± 0.16) was significantly higher than the control N2a tumors (0.075 ± 0.011; P < 0.002) for the five mice. The mean FDG %ID/g values of tk-rl-expressing and -control tumors were not significantly different as expected (2.45 ± 0.25 versus 2.6 ± 0.18; Fig. 3A). Although the cell culture data showed a decrease in TK activity with tk-rl fusion in comparison to HSV1-sr39tk, microPET imaging reveals easily detectable FHBG accumulation in the tumors expressing the TK-RL fusion protein.
The tumor formed by 

signal of the tk20rl expressing tumors were well correlated (R² = 0.89) across the eight mice (Fig. 3B). The TK and RL activities of these four stable clones in vitro were also well correlated (R² = 0.91; data not shown).

Renilla Luciferase Reporter Gene Expression Can Be Serially Measured in Cells Stably Expressing the tk20rl Fusion Construct in Living Mice with High Sensitivity. One of the greatest advantages of optical bioluminescence imaging is its comparatively high sensitivity (allowing detection of low cell numbers) for imaging gene expression, whereas microPET imaging requires a greater tumor volume (3–5 mm) or mass of cells to obtain a detectable signal. We therefore implanted tk20rl-expressing and -nonexpressing control N2a cells as tumors on both the shoulders of four nude mice and imaged them daily using a cooled CCD camera to monitor the expression level of the fusion reporter construct. Significant signal is not seen on the first day (both the control N2a and tk20rl-expressing tumors showed bioluminescence value maximum at ~4 × 10⁸ p/sec/cm²/sr), but after the second day, the optical signal in the tk20rl-expressing tumors started increasing progressively and reached a maximum of 6 × 10⁶ ± 1.5 × 10⁶ p/sec/cm²/sr on day 10, whereas the signal in control tumors remained unchanged (12.0 × 10⁴ ± 4.2 × 10⁴ maximum p/sec/cm²/sr) throughout the study (Fig. 4). With the gradual increase in rl expression, we observed gradual growth of the tumors that attained a diameter of ~0.6–0.8 cm at day 10. We also attempted microPET imaging of these mice when the tumors were not palpable but were unable to obtain any detectable level of signal (data not shown). Presence of the bioluminescence rl reporter gene in the tk20rl fusion construct, therefore, confers a highly sensitive tool for monitoring reporter gene expression.

Discussion

In this study, we report the construction of a novel fusion gene vector harboring HSV1-sr39 thymidine kinase, a PET reporter gene and renilla luciferase, a bioluminescence reporter gene and validate its application in living mice using two different imaging modalities.

Fig. 3. In vivo imaging of tk20rl fusion protein by using two different modalities. A, optical and microPET imaging of tk20rl fusion construct in the same nude mouse. A total of 2 × 10⁶ of N2a cells stably expressing the tk20rl fusion construct and control N2a cells was implanted s.c. on left and right shoulders in a nude mouse. After 4 days when each tumor was ~3–4 mm in diameter, the mouse was first scanned in the CCD camera after injection of coelenterazine via tail vein and bioluminescence signal was recorded as maximum p/sec/cm²/sr (left panel). The same mouse was imaged 4 days later by microPET using FHBG (center panel) and again using FDG on the following day (right panel). The tumor formed by tk20rl expressing cells shows high bioluminescence as well as FHBG accumulation in comparison to the control tumor. The FDG image represents the viability of both tk20rl and control tumor. Non-specific accumulation of tracer was found in the gastrointestinal tracts (GI), bladder in case of FHBG (attributable to clearance of tracer), and in GI tract and brain in case of FDG (attributable to high metabolic activity). B, in vivo correlation of TK and RL gene expression exhibited by four clones of N2a cells stably but differentially expressing the tk20rl fusion. A total of 2 × 10⁶ cells of each of four clones were implanted on the left shoulders of two nude mice each, and after 8–10 days, mice were imaged by microPET and optical CCD camera on the same day. Plot of %ID/g of FHBG versus bioluminescence signal as expressed as maximum (p/sec/cm²/sr) obtained from the ROI drawn on the tumors of images (r² = 0.89). Each of the eight data point represents ROI data from the bioluminescent and PET image of the same mouse.

Fig. 4. Imaging serial increase in rl gene expression over time in tumors stably expressing the tk20rl fusion. A total of 2 × 10⁶ of N2a cells stably expressing tk20rl fusion gene and control N2a cells was implanted on the left and right shoulders, respectively, of a single nude mouse and imaged daily using the optical CCD camera after injection of coelenterazine. A gradual increase in bioluminescence was observed in the tumor expressing tk20rl fusion over time but not in the control tumor.
HSV1-thymidine kinase (both wild-type and the sr39 mutant) are well-established PET reporter genes (7, 11, 12) for imaging gene expression. Fusion vectors harboring wild-type tk and green gfp (tk-gfp) constructed by several groups (16, 17) could retain sufficient levels of TK activity and be imaged by microPET. As shown herein, the sr39thymidine kinase-renilla luciferase fusion vectors constructed by us could also maintain the TK and RL activities in different cell lines at different levels. However, our attempt to build a fusion construct of tk and fl yielded a poorly active fusion protein (unpublished data). Another triple fusion construct bearing wild-type tk, fl, and neomycin genes (tk-fl-neo; Ref. 18) also showed very low TK activity in comparison to the tk vector alone in cell culture in our hands (unpublished data). Therefore, the nature of the fusion partner also affects the activity of the TK enzyme. Moreover the length of the spacer between the two proteins seems to play an important role in maintaining functionality of each protein, which has also previously been reported for various fusion constructs (16). In addition, it is likely that the a.a. sequence and composition of the spacer can influence the activities of either enzyme. The order of the fusion partners of the construct might also influence the activities of the proteins depending on the positioning of critical amino acids in the protein backbones. It has recently been shown that small changes in HSV1-tk, including several critical amino acids at the COOH-terminal end (19), make this enzyme more prone to loss in activity, suggesting care is needed in fusing proteins to the COOH terminus. The tk2-fl fusion described in the current work has a decreased TK activity that might be improved by using a longer/different spacer between the two genes or placing tk as the downstream gene. On the other hand, the tk2-fl fusion exhibits ~6–8-fold increase in RL activity in comparison to the rl alone that has made this fusion vector superior for bioluminescence imaging. However, a true comparison of the activities of the fusion protein with the nonfused control proteins can be made only after measuring the $K_m$ and $V_{max}$ for each protein. Future studies will need to purify each protein and study the substrate kinetics in a detailed fashion to better understand the effects of fusing the individual proteins on the proteins ability to act on substrate.

Our results demonstrate that despite decreased TK activity, it is possible to image the TK2-RL fusion protein noninvasively and repetitively in living mice both by microPET and by an optical CCD camera. Therefore, this fusion reporter gene has the potential to translate approaches from small animal models to preclinical and clinical applications. We know of only one report in the literature on measuring bioluminescence at the single cell level using a similar setup. However, this approach might not be as useful as fluorescence approaches for cell reporter at the single cell level using a similar setup. However, this approach often require special surgical procedures for exposing the animals. In contrast, we can easily detect and quantitatively and reproducibly evaluate the bioluminescence signal from various sites within the intact living mouse as described in our previous reports (4, 5).

The fusion reporter gene described here may have some limitations because of partial cleavage (~25–35%) of the fusion into its two component proteins, which might result in a loss of sensitivity in different cell lines depending on the presence of specific proteases. However, the high correlation of TK and RL activities of the stably expressing cell lines in both in vitro and in vivo suggest that this fusion will be useful in monitoring tumor growth and cell trafficking studies where steady-state expression is expected. Future studies will need to explore alternate spacers to minimize cleavage of the fusion protein.

The higher sensitivity of optical imaging allows lower levels of reporter gene expression and/or lower numbers of expressing cells to be imaged relative to the PET approach. The sensitivity differences cannot be accounted for because of the reduced TK activity alone because this would only account for a 3–5-fold difference, and the number of cells detectable by optical imaging are several log-fold lower. We could follow reporter gene expression level using the fusion protein from a very early stage of s.c. implanted cells using the cooled CCD camera. Additional studies will be needed to better characterize the differences in sensitivity at various depths within a mouse. However, the drawback of bioluminescence imaging is this approach is not tomographic and difficult to translate into humans. Presence of the PET reporter gene in this fusion protein, on the other hand, is compatible with tomographic tools for measuring reporter gene expression that could also be used in larger subjects including humans. This fusion protein, therefore, provides a unique tool of validating different approaches quickly in small animal models at a very low number of cells that can be rapidly translated to clinical use. Future use of this fusion, including single cell imaging, should foster additional implementation of reporter genes directly from the cell to animal to human level. This, in turn, should lead to acceleration of many areas of cancer research, including cell trafficking, tumor therapy, and gene therapy.

Acknowledgments

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References


\footnote{Ray, P., Min, J. J., Gambhir, S. S., Multimodality imaging of a fusion reporter gene. 4 Ray, P., Min, J. J., Gambhir, S. S., Multimodality imaging of a fusion reporter gene.}


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