Escherichia coli Nissle 1917 Facilitates Tumor Detection by Positron Emission Tomography and Optical Imaging

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Abstract

Purpose: Bacteria-based tumor-targeted therapy is a modality of growing interest in anticancer strategies. Imaging bacteria specifically targeting and replicating within tumors using radiotracer techniques and optical imaging can provide confirmation of successful colonization of malignant tissue.

Experimental Design: The uptake of radiolabeled pyrimidine nucleoside analogues and \(^{[18F]} FDG\) by \textit{Escherichia coli} Nissle 1917 (EcN) was assessed both \textit{in vitro} and \textit{in vivo}. The targeting of \textit{EcN} to 4T1 breast tumors was monitored by positron emission tomography (PET) and optical imaging. The accumulation of radiotracer in the tumors was correlated with the number of bacteria. Optical imaging based on bioluminescence was done using \textit{EcN} bacteria that encode luciferase genes under the control of an L-arabinose – inducible PBAD promoter system.

Results: We showed that \textit{EcN} can be detected using radiolabeled pyrimidine nucleoside analogues, \(^{[18F]} FDG\) and PET. Importantly, this imaging paradigm does not require transformation of the bacterium with a reporter gene. Imaging with \(^{[18F]} FDG\) provided lower contrast than \(^{[18F]} FEAU\) due to high FDG accumulation in control (nontreated) tumors and surrounding tissues. A linear correlation was shown between the number of viable bacteria in tumors and the accumulation of \(^{[18F]} FEAU\), but not \(^{[18F]} FDG\). The presence of \textit{EcN} was also confirmed by bioluminescence imaging.

Conclusion: \textit{EcN} can be imaged by PET, based on the expression of endogenous \textit{E. coli} thymidine kinase, and this imaging paradigm could be translated to patient studies for the detection of solid tumors. Bioluminescence imaging provides a low-cost alternative to PET imaging in small animals.

In recent years, successful targeting of viruses and bacteria to solid tumors has been shown (1, 2) and such oncolytic therapy is receiving renewed interest. Tumor-targeting bacteria have been studied and they showed preferential accumulation in tumors compared with normal organs; studies have included the use of \textit{Bifidobacterium} spp. (3), \textit{Listeria monocytogenes} (1, 4), \textit{Clostridium} spp. (5), \textit{Salmonella} spp. (6–8), \textit{Shigella flexneri} (6), \textit{Vibrio cholerae} (2), and \textit{Escherichia coli} (6). A number of different oncolytic viruses have already entered into clinical trials and adenovirus H101 has been approved in China for the treatment of head and neck cancer (8). However, only a single phase I clinical trial using bacteria, \textit{Salmonella} VNP20009, has been initiated (7). In this trial, a lower percentage of tumor-targeting efficacy was observed compared with the previously investigated rodent models in which tumor-colonization was high (7). The authors stated that this discrepancy could be the result of inadequate sampling that was inherent in their use of fine-needle biopsies. In an excisional biopsy done on one patient, bacteria were found to colonize the tumor, whereas a previous needle biopsy of the same tumor did not detect the microorganisms. Currently, biopsy is the only clinical method available for determining the presence of bacteria. Future clinical studies will require the ability to accurately detect the presence of bacteria in tumors (and also in other organs and tissues) without excision of the respective tissue.

To address this issue, noninvasive imaging of bacteria-colonized tumors has several advantages compared with biopsy. In contrast to biopsies, imaging can be done repeatedly, provides a much wider assessment of the entire tumor as well as other tissues and body organs (i.e., minimizes sampling errors), and can provide both a spatial and time dimension from sequential tomographic images.

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Different imaging modalities [positron emission tomography (PET), single-photon emission computed tomography, and optical imaging] in combination with reporter genes have been used to visualize the distribution of microorganisms and to confirm their presence within experimental tumors. Most studies on bacterial tumor colonization in tumor-bearing mice have used luciferase and/or fluorescence (green fluorescent protein) imaging for bacterial detection (2, 4, 6, 9). However, current optical imaging modalities using fluorescent proteins or luciferases are restricted to small animals and cannot be readily translated to patient studies. Therefore, radiotracer or magnetic resonance imaging techniques need to be used to track bacteria in human subjects.

The best known and most widely used radiotracer for PET imaging is fluorine-18 ([18F]–labeled fluorodeoxyglucose ([18F]FDG), which is accumulated by metabolically active cells. On entry into the cell, [18F]FDG is phosphorylated by hexokinase; the phosphorylated FDG can neither exit the cell nor be further metabolized and is therefore trapped within the cell in relation to the level of glycolytic activity. FDG uptake in many malignant tumors is high because glucose metabolism in the tumors is high. In addition, any inflammatory processes associated with the tumor contribute to the high FDG uptake because granulocytes and macrophages also have high rates of glucose metabolism (10). Although tumor tissue targeted by bacteria is likely to have high levels of FDG accumulation, baseline (before bacterial administration) is also likely to be high, and the difference between baseline and tumor-targeted FDG uptake may be difficult to image and quantitate.

Another powerful imaging strategy is the use of reporter genes in to identify the location and number of tissue-targeted bacteria. Among the PET-based reporter genes, herpes simplex virus 1 thymidine kinase (HSV1-TK) has been used most extensively. The expression of HSV1-TK can be imaged and monitored using specific radiolabeled substrates that are selectively phosphorylated by HSV1-TK and trapped within transfected cells. [18F]-2'-Fluoro-2'-deoxy-1β-D-arabinofuranosyl-5-ethyl-uracil ([18F]FEAU) and [124I]2'-fluoro-1β-D-arabinino-furanosyl-5-ido-uracil ([124I]FIAU) are radiopharmaceuticals for imaging HSV1-TK gene expression (11) and are used widely by many investigators (12–15). HSV1-TK–expressing Salmonella VNP20009 have recently been shown to localize in tumors, including s.c. Salmonella (12–15). HSV1-TK has been used in human subjects.

In contrast to using an exogenous reporter gene such as HSV1-TK, we investigated the feasibility of using the endogenous thymidine kinase of probiotic E. coli Nissle 1917 (EcN) to phosphorylate [18F]FEAU and [124I]FIAU for noninvasive PET imaging of EcN-colonized tumors.

We show that the uptake of [18F]FEAU by the tumors is dependent on the presence of EcN and that the magnitude of radioactivity accumulation correlates with the number of bacteria that colonize the tumor. We also compared [18F]FEAU and [124I]FIAU images to those obtained with [18F]FDG. Bioluminescence images of EcN were also obtained and the optical signal shown to colocalize with the [124I]FIAU activity distribution in the same animals, showing the feasibility of using EcN for identifying tumors by both bioluminescence and PET imaging in small animals.

**Materials and Methods**

**Cell culture and animal experiments**

The murine mammary carcinoma cell line 4T1 (ATCC CRL-2539) was cultured in RPMI containing 10% FCS. The cells were maintained at 37°C with 5% CO2 in air, and subcultured twice weekly. For tumor cell implantation, 6- to 8-week-old athymic nu/nu mice (National Cancer Institute) were used, housed five per cage, and allowed food and water ad libitum in the Memorial Sloan Kettering Cancer Center facility for 1 wk before tumor cell implantation. The 4T1 cells were removed by trypsinization, washed in PBS, and 3.3 × 10^4 cells (resuspended in 50-μL PBS) were implanted s.c. into the right and left shoulders. Two weeks postimplantation (tumor diameter >5 mm), bacteria were administered systemically by tail vein injection.

Animal studies were done in compliance with all applicable policies, procedures, and regulatory requirements of the Institutional Animal Care and Use Committee, the Research Animal Resource Center of Memorial Sloan Kettering Cancer Center, and the NIH Guide for the Care and Use of Laboratory Animals. All animal procedures were done by inhalation of 2% isofluorane. After the studies, all animals were sacrificed by CO2 inhalation.

**Bacteria**

E. coli Nissle 1917 (EcN), a probiotic, non–protein-toxin-expressing strain, was used to specifically colonize tumors and harbored a pBR322DEST P_HAS-DUAL-term, a luxABCD-EDE-luciferase encoding plasmid that enables the bacteria to be detected with bioluminescence imaging when induced with 1-arabinose (6). The light is emitted from the bacteria as a result of a heterodimeric luciferase (encoded by luxAB) catalyzing the oxidation of reduced flavin mononucleotide and a long-chain fatty aldehyde (synthesized by a fatty acid reductase complex encoded by luxCDE; ref. 17). For i.v. injection, bacteria were grown in LB broth supplemented with 100 μg/mL ampicillin until reaching an absorbance at 600 nm (A_600 nm) of 0.4 (corresponding to 2 × 10^8 colony-forming units (CFU)/mL) and washed twice in PBS. The suspension was then diluted to 4 × 10^7 CFU/mL and 100 μL were injected into the lateral tail vein of tumor-bearing mice. Vehicle control mice were injected with 100-μL PBS via tail vein.

**Radiopharmaceuticals**

[18F]FEAU was synthesized by coupling the radiolabeled fluoro sugar with the silylated pyrimidine derivatives following a procedure previously reported by Seganova et al. (12). The specific activity of the product was >57 GBq/μmol (~1 Ci/μmol); radiochemical purity was >95% following purification by high-pressure liquid chromatography. [124I]FIAU was synthesized by reacting the precursor of 5-trimethyl-stannyl 1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]uracil (FTAU) with carrier-free [124I]NaI. 1-124 was produced on the Memorial Sloan Kettering Ecbo cyclotron using the 124Te(p,n) 124I nuclear reaction on an enriched 124TeO2/Al2O3 solid target. Radiosynthesis was done as previously described (13, 14) with minor modifications. The specific activity of the product was >1,000 GBq/μmol (~27 Ci/μmol); radiochemical purity was >95% and was determined by radio TLC (R_f 0.7) using silica gel plates and a mobile phase of ethyl acetate/acetone/water (14:8:1, v/v/v).

[18F]FDG (clinical grade) was obtained from IBA Molecular with a specific activity >41 MBq/μmol (~11 μCi/μmol) and a radiochemical purity of 99% by TLC and 98% by high-pressure liquid chromatography.

**In vitro uptake of [18F]FDG and [18F]FEAU**

An overnight culture of EcN was diluted 1:50 into 5-mL fresh LB broth containing either 0.925 MBq (25 μCi) of [18F]FDG or [18F]FEAU and grown at 37°C for 4 h. The bacteria were then harvested by centrifugation, washed twice with PBS, and the radioactivity in the pelleted bacteria and medium was measured in a gamma counter (Packard, United Technologies).
MicroPET imaging

**FDG.** In the first group of six animals, each animal was injected via the tail vein with 9.25 MBq (250 μCi) of $[^{18}F]$FDG before and 16 or 72 h after administration of EcN. $[^{18}F]$FDG PET scanning was done 1 h after tracer administration using a 10-min list mode acquisition. Animals were fasted 12 h before tracer administration and kept under anesthesia between FDG injection and imaging.

**FEAU.** In the second group of 24 animals, three subgroups of eight animals each were studied; each animal was injected via tail vein with 9.25 MBq (250 μCi) of $[^{18}F]$FEAU. Subgroup 1 (control) was not injected with bacteria (they received 100-μL PBS); subgroups 2 and 3 were injected with EcN bacteria 16 and 72 h before $[^{18}F]$FEAU administration. $[^{18}F]$FEAU PET scanning was done 2 h after tracer administration using a 10-min list mode acquisition.

**FIAU.** In a third set of six mice, three were injected with EcN bacteria and three with PBS (control). $[^{124}I]$FIAU (37 MBq (1 mCi)) was injected in each animal i.v. 72 h after bacterial injection. Potassium iodide was used to block the uptake of radioactive iodine by the thyroid. $[^{124}I]$FIAU PET was obtained 4, 8, 12, 24, 48, and 72 h after tracer administration with 10-min list acquisition at the 4- and 8-h imaging time points, 15 min at the 12-h time point, 30 min at 24 h, and 60 min at the 48- h and 72-h time points. After tracer administration and between imaging time points, the animals were allowed to wake up and maintain normal husbandry.

Imaging was done using a Focus 120 microPET dedicated small-animal PET scanner (Concorde Microsystems, Inc.). Mice were maintained under 2% isoflurane anesthesia with an oxygen flow rate of 2 L/min during the entire scanning period. Three-dimensional list mode data were acquired using an energy window of 350 to 700 keV for $^{18}F$ and 410 to 580 keV for $^{124}I$ and a coincidence timing window of 6 ns. These data were then sorted into two-dimensional histograms by Fourier rebinning using a span of 3 and a maximum ring difference of 47. Transverse images were reconstructed by filtered back-projection using a ramp filter with a cutoff frequency equal to the Nyquist frequency in a 128 × 128 × 94 matrix composed of 0.866 × 0.866 × 0.866-mm voxels. The image data were corrected for (a) nonuniformity of scanner response using a uniform cylinder source-based normalization, (b) dead time count losses using a singles count rate–based global correction, (c) physical decay to the time of injection, and (d) the 124I branching ratio. There was no correction applied for attenuation, scatter, or partial-volume averaging. The count rates in the reconstructed images were converted to activity concentration [percent of injected dose per gram of tissue (%ID/g)] using a system calibration factor (μCi/mL/cps/voxel) derived from imaging of a rat-size phantom filled with a uniform aqueous solution of $^{18}F$.

PET image analysis was done using ASIPro software (Concorde Microsystems, Inc.). For each PET scan, regions of interest were manually drawn over tumor, liver, skeletal muscle, and heart. For each tissue and time point postinjection, the measured radioactivity was expressed as %ID/g. The maximum pixel value was recorded for each tissue and tumor-to-organ ratios for liver, skeletal muscle, and heart were then plotted versus time.

Bacterial and radioactivity quantification of tissue samples

Euthanized mice were rinsed with 100% ethanol before tissue removal. Organs such as liver, lung, spleen, and heart were sampled and weighed before radioactivity measurements. Tumor tissue was weighed and homogenized in 1-mL PBS. Serial dilutions of the homogenized sample were plated on L-arabinose–containing LB agar plates and growing colonies were counted and confirmed to be EcN harboring a pBpR322DEST PBAD-DUAL-term by bioluminescence imaging using an IVIS 100 Imaging System (Caliper). The remaining tumor suspension was assayed for radioactivity in a gamma counter (Packard, United Technologies). $[^{18}F]$FEAU radioactivity (%ID/g) in the samples was determined and tumor-to-organ ratios were calculated. To assess the correlation between radioactivity and scintillation counter measurements, the Pearson correlation coefficient was computed.

In vivo optical imaging of bioluminescence

The same animals were imaged for localization of bioluminescence after the 72-h $[^{124}I]$FIAU PET scans. Each animal was injected with 200-μL L-arabinose (25% w/v) to induce transcriptional expression of the luciferase reporter for bioluminescence imaging. Images were acquired for 60 s, 4 h after L-arabinose injection, using an IVIS 100 Imaging System (Caliper). The photon emissions (photons/cm²/s/steradian) from the animals and cell samples were analyzed using the LIVINGIMAGE 2.5 software (Caliper).

Statistics

A two-tailed unpaired t test was applied to determine the significance of differences between values using the MS Office 2003 Excel 11.0 statistical package (Microsoft).

Results

In vitro $[^{18}F]$FDG and $[^{18}F]$FEAU uptake into EcN. The in vitro uptakes of $[^{18}F]$FDG and of $[^{18}F]$FEAU by the tumor-colonizing strain *E. coli* Nissle 1917 were compared. There was a 120-fold higher concentration of $[^{18}F]$FDG and a 6.5-fold higher concentration of $[^{18}F]$FEAU activity in EcN-bacteria compared with that in the LB broth, suggesting that $[^{18}F]$FDG would be a better imaging agent than $[^{18}F]$FEAU.

Distribution of EcN in tumor-bearing mice. Following EcN injection into the tail vein of 4T1 tumor-bearing mice, most bacteria (>99%) are quickly cleared from the animals and only a small percentage of the administered bacteria colonize the tumor (6). These tumor-colonizing bacteria started to grow exponentially for ~24 hours before reaching a plateau of $1 \times 10^9$ CFU/g of tumor tissues. During the growth phase, the bacteria are metabolically active and rapidly proliferate. For our studies, we elected to use tumor-bearing mice that were injected with EcN at 16 hours (lower CFU per gram but in rapid growth phase) and at 72 hours (higher numbers of bacteria in a slower phase) before administration of $[^{18}F]$FDG or $[^{18}F]$FEAU. The number of bacteria per gram of tumor tissue at 16 and 72 hours postinjection is shown in (Fig. 1).

In vivo PET imaging of EcN colonized tumors. $[^{18}F]$FDG PET imaging was done before and at 16 and 72 hours after tail vein injection of EcN in the same animals (Fig. 2A). The

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**Fig. 1.** Tumor colonization of EcN at 16 and 72 h after bacterial injection. Columns, mean of eight analyzed tumors; bars, SD.
[18F]FDG tumor-to-organ ratios (mean ± SD) before injection of EcN bacteria were high in liver (1.6 ± 0.4) and muscle (3.3 ± 0.7) and low in heart (0.23 ± 0.12). At 16 hours after EcN injection, tumor-to-organ ratios were significantly increased for liver, muscle, and heart (2.6 ± 0.5, 5.4 ± 1.1, and 0.31 ± 0.8, respectively). At 72 hours after EcN injection, the tumor-to-organ ratios were lower for the same tissues (2.2 ± 0.8, 4.3 ± 1.2 and 0.31 ± 0.17, respectively). This represents a ~1.7-fold enhancement at 16 hours (P < 0.0001 for liver and muscle, P < 0.06 for heart), but only a ~1.4-fold enhancement at 72 hours (P < 0.05 for liver, muscle, and heart). Although the post-EcN ratios were significantly higher than the pre-EcN ratios at 16 and 72 hours, the magnitude of this change and contrast in the images was relatively small (Fig. 2A). Furthermore, the decrease in the activity concentration ratio from 16 to 72 hours does not reflect the increase in bacteria over this time period (Fig. 1).

[18F]FEAU PET imaging was also done before and at 16 and 72 hours after EcN tail-vein injection in separate sets of animals (Fig. 2C). The uptake of [18F]FEAU in tumors was much lower than that of [18F]FDG before EcN injection and the tumor-to-organ ratios were lower than those obtained with FDG (except for heart), ranging at 1.4 ± 0.3 for liver, 1.4 ± 0.3 for heart, and 1.7 ± 0.4 for muscle. After injection of bacteria, the tumor-to-organ ratios were substantially higher, reaching values of 2.0 ± 0.7 at 16 hours and 2.4 ± 0.8 at 72 hours for liver, 2.6 ± 0.8 at 16 hours and 3.0 ± 1.3 at 72 hours for muscle, and 2.1 ± 0.6 at 16 hours and 2.9 ± 0.8 at 72 hours for heart. This represents a ~1.6-fold enhancement at 16 hours (P < 0.001) and a ~2.1-fold enhancement at 72 hours (P < 0.0002). Here, the greater enhancement in the tumor-to-organ ratios is consistent with the greater contrast seen in the images (Fig. 2C).

Although the [18F]FDG tumor-to-organ enhancement ratios were much more variable than those for [18F]FEAU, there was no statistically significant difference between FDG and FEAU at 16 hours after EcN injection. However, the enhancement ratio after EcN injection was significantly higher (P < 0.05) for [18F]FEAU compared with [18F]FDG at the 72-hour time point, and this is clearly seen in the images (Fig. 2A and C).

**Tissue sampling.** In a separate tissue sampling set of experiments, measurements of tumor-to-organ (liver, spleen, heart, and lung) [18F]FEAU radioactivity ratios were obtained (Fig. 3). In all tissues assayed, the tumor-to-organ ratios increased significantly (P < 0.02) after EcN administration. The higher tumor-to-organ ratios at 72 hours compared with 16 hours (Fig. 3) corresponded with higher numbers of bacteria in the tumors at 72 hours compared with 16 hours (Fig. 1).

**Correlation between number of bacteria and [18F]FEAU uptake.** Tumor radioactivity (%ID/g) was plotted versus the number of bacterial CFUs per gram for each of the tumors imaged in Fig. 2C and D data set, and a linear correlation (Pearson coefficient, R² = 0.909) was obtained (Fig. 4A and B). This indicates that the [18F]FEAU uptake (%ID/g) reflects the number of EcN bacteria that have colonized the 4T1 xenografts.

**Time course study of [124I]FIAU distribution.** To determine whether other thymidine analogues can be used to image bacteria, two groups of 4T1 xenograft-bearing mice were injected with EcN bacteria or PBS (control) 72 hours before i.v.
administration of $^{124}$I[FIAU]. PET imaging was done 4, 8, 12, 24, 48, and 72 hours after $^{124}$I[FIAU] administration (Fig. 5A). The time course of $^{124}$I[FIAU] was included to show the advantage of "later-time imaging" with $^{124}$I[FIAU] in achieving better target-to-background images. I-124 has a radioactive half-life of 4 days, whereas F-18 has a half-life of 110 minutes. Images from the 4- and 8-hour acquisition are not shown due to very high background radioactivity at these time points. By 48 and 72 hours, background $^{124}$I[FIAU] activity has cleared substantially and the tumor-to-background ratios are >5 in the EcN-treated animals (Fig. 5B). However, the control (non–EcN-treated) animals also show some $^{124}$I[FIAU] retention in the 4T1 xenografts. This reduces the specificity of the radioactivity measured in the EcN-treated tumors and results in only a 2.5-fold enrichment of $^{124}$I[FIAU] in the bacteria-treated tumors (Fig. 5B).

**Colocalization of bioluminescence and $^{124}$I[FIAU] uptake.** To further verify that the increased $^{124}$I[FIAU] PET signal reflected bacterial localization in 4T1 xenografts, we took advantage of the t-arabinose–inducible luciferase reporter plasmid pBR322DEST P$_{BAD}$-DUAL-term (6). t-Arabinose was injected into each mouse following $^{124}$I[FIAU] PET imaging, and bioluminescence imaging was done 4 hours later when the expression of luciferase is at its maximum (6). The t-arabinose–induced bioluminescence signal was readily detected at the site of the 4T1 xenografts (Fig. 5C). Control tumors did not show any such signal. The bioluminescence images of EcN-treated mice also indicated no bacterial presence in other tissues of mice.

**Discussion**

EcN is one of the best studied probiotic bacterial strains and it has been successfully used in humans as an oral treatment for a number of intestinal disorders (e.g., diarrhea, inflammatory bowel diseases, and ulcerative colitis) for more than 90 years (18, 19). Although the genome of EcN shows high similarity to the uropathogenic E. coli CFTR073 (20), the probiotic strain lacks any known protein toxins or mannose-resistant hemagglutinating adhesins (21). Furthermore, EcN was not found to colonize any organs other than tumor when administered systemically to tumor-bearing mice (6). Thus, EcN seems to be a good candidate for human application, although it still produces lipopolysaccharide (endotoxin), which could result in adverse effects. Because deletion of genes responsible for lipopolysaccharide biosynthesis (e.g. msbB) has been shown to be successful for Salmonella typhimurium, a similar strategy could be adopted with EcN to insure its clinical safety.

A noninvasive, clinically applicable method for imaging bacteria in target tissue or specific organs of the body would be of considerable value for monitoring and evaluating bacterial-based therapy in human subjects. This imaging system could also be used for monitoring the targeting and proliferation of the bacterial vector, such as EcN, to identify sites of occult tumor and to identify sites of bacterial proliferation in occult infectious disease. EcN imaging provides the following benefits: Following systemic administration of the bacteria,
imaging can (a) confirm successful targeting to known tumor sites, (b) potentially identify additional sites of tumor metastases, and (c) assess whether the number (concentration) of EcN in tumor tissue is adequate to deliver a sufficient dose of a “therapeutic gene.” In our study, we assessed the feasibility of detecting EcN-colonized tumors with $^{[18}\text{F}]$FDG, $^{[18}\text{F}]$FEAU, and $^{[124}\text{I}]$FIAU PET imaging.

We showed that EcN accumulate and trap radiolabeled $^{[18}\text{F}]$FDG, $^{[18}\text{F}]$FEAU, and $^{[124}\text{I}]$FIAU using endogenous enzyme systems (e.g., bacterial hexokinase and thymidine kinase). It was previously shown that tumor targeting of HSV1-TK–transformed Salmonella VNP20009 could be successfully imaged with $^{[124}\text{I}]$FIAU and that $^{[124}\text{I}]$FIAU accumulation was HSV1-TK dependent (16). Here, the expression of the endogenous bacterial thymidine kinase of EcN and phosphorylation of $^{[18}\text{F}]$FEAU and $^{[124}\text{I}]$FIAU are sufficient to result in selective accumulation of these radiotracers in tissue colonized by EcN. In contrast to the marked structural specificity of mammalian thymidine kinase for thymidine alone (resulting in little or no phosphorylation of thymidine analogues), the thymidine kinase of bacteria has been shown by Bettegowda et al. (5) to be less specific for thymidine than the mammalian enzyme. Bacterial as well as viral thymidine kinase will phosphorylate thymidine analogues such as FIAU and FEAU. This study opens up new possibilities for future investigations and for the use of alternative pyrimidine nucleoside derivatives such as FEAU that can be selectively phosphorylated by endogenous bacterial thymidine kinase (e.g., E. coli, Salmonella, or Clostridium).

The tumor-selective replication of EcN in live animals allowed us to distinguish tumors from other tissues by PET imaging following administration of radiolabeled $^{[18}\text{F}]$FEAU or $^{[124}\text{I}]$FIAU. By using tumors in different stages of bacterial colonization (i.e., 16 and 72 hours after bacterial administration), we showed a linear relationship between the number of viable bacteria in tumor tissue and the uptake of radiolabeled $^{[18}\text{F}]$FEAU. This result is similar to that found with HSV1-TK–transformed Salmonella VNP20009 and $^{[124}\text{I}]$FIAU accumulation (16). Comparing the Salmonella VNP20009 and EcN data shows that the HSV1-TK–transformed Salmonella accumulate more radiopharmaceutical per viable bacteria than EcN bacteria over the dose ranges that were studied (Fig. 4B). These results, for several reasons, are not unexpected and indicate that there is a role for reporter-transformed bacteria when higher imaging sensitivity is required: In addition to the genomic thymidine kinase gene of Salmonella VNP20009, HSV1-TK was present in multiple copies under control of a constitutive promoter. In contrast, only the genomic copy of the EcN thymidine kinase gene under control of its own promoter was present in EcN bacteria. Therefore, higher expression of thymidine kinase is achieved in VNP20009 Salmonella. Furthermore, $^{[124}\text{I}]$FIAU and $^{[18}\text{F}]$FEAU were developed to specifically image HSV1-TK, and not mammalian TK1, to achieve low background activity, and
these tracer substrates may not be an ideal substrate for bacterial thymidine kinases (5).

There was no correlation between the level of $[^{18}F]$FDG uptake and number of viable bacteria in the tumors, and the signal-to-background ratio was not as high with $[^{18}F]$FDG as with $[^{18}F]$FEAU and $[^{124}I]$FIAU. This clearly reflects the high baseline uptake (95±D/g) of $[^{18}F]$FDG by the tumor compared with that of $[^{18}F]$FEAU and $[^{124}I]$FIAU. However, $[^{18}F]$FDG imaging in combination with EcN (or other bacteria) might show better results in tumors with a low baseline level of $[^{18}F]$FDG uptake. The absence of a correlation between number of viable bacteria and $[^{18}F]$FDG uptake might also be due to the presence of necrosis induced by the bacteria or to the presence of glucose-metabolizing macrophages in the tumors (6). For example, on day 1 after bacterial injection, a high number of metabolically active bacteria were present and only very small patches of necrosis were observed. Two days later, the number of bacteria increases, but the number of living cells in the tumor decreases dramatically because the necrotic region takes up 30% to 50% of the tumor volume (6).

It should also be noted that 4T1 xenografts in the absence of bacteria accumulate $[^{124}I]$FIAU to low levels above background (48-and 72-hour images in Fig. 5B) in comparison with the near-background levels of $[^{18}F]$FEAU accumulation (Fig. 2D) in non–bacteria-treated animals. This is consistent with similar observations in other tumor systems (12–14, 22, 23). Thus, $[^{18}F]$FEAU may be a better bacterial-imaging probe than $[^{124}I]$FIAU.

The current study showed the feasibility of noninvasive imaging of bacteria based on the expression of genomic bacterial thymidine kinase. The potential for monitoring patients that have received tumor-colonizing bacteria without the inclusion of an exogenous (e.g., viral) reporter gene has previously been shown (5) and is confirmed here. Imaging should be able to determine whether bacterial tumor colonization has occurred successfully and whether previously undetected metastases or specific organs are colonized by the bacteria. We have shown that the level of radioactivity can also be taken as an indicator of the number of bacteria that are present in the target tissue and whether therapeutic effects (e.g., by administration of prodrugs or induction of toxic genes) can be expected. In addition, the presence of pathogenic bacteria in localized infections may also be identifiable, and it may also be possible to differentiate bacterial infections from nonmicrobial inflammations by $[^{18}F]$FEAU or $[^{124}I]$FIAU PET imaging.

In conclusion, the results of our study indicate that EcN (or other bacteria expressing endogenous thymidine kinase) can be imaged with pyrimidine nucleoside analogues that are selectively phosphorylated and trapped in the bacteria. The advantage of using EcN over many other bacteria is their probiotic character. It is therefore a relatively safe “imageable vector” that could also include genes conferring therapeutic potential. We show that the PET images for EcN-colonized tumors were better (i.e., resulted in higher signal-to-background ratios) with $[^{18}F]$FEAU than with $[^{18}F]$FDG, and this was mainly due to the low baseline (pre-bacterial) activity in the tumors and surrounding tissue. Most importantly, a linear relationship between the number of viable bacteria and level of $[^{18}F]$FEAU activity in the xenografts was found, an essential component of the imaging paradigm. Other pyrimidine nucleoside analogues that have been developed for PET imaging of HSV1-TK, such as $[^{124}I]$FIAU and $[^{18}F]$FBG, could also be further evaluated for noninvasive monitoring of bacterial tumor colonization because both positron-emitting radiopharmaceuticals have already been successfully administered to patients in gene imaging studies (15, 23–26).

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References

Escherichia coli Nissle 1917 Facilitates Tumor Detection by Positron Emission Tomography and Optical Imaging

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