In vivo Tumor Targeting Using a Novel Intestinal Pathogen-Based Delivery Approach

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Abstract

Efficient methods for tumor targeting are eagerly awaited and must satisfy several challenges: molecular specificity, transport through physiologic barriers, and capacity to withstand extracellular or intracellular degradation and inactivation by the immune system. Through interaction with its hosts, the intestinal pathogen-produced Shiga toxin has evolved molecular properties that are of interest in this context. Its nontoxic B-subunit binds to the cellular toxin receptor, glycosphingolipid Gb3, which is highly expressed on human cancers and has recently been reported to be involved in the formation of metastasis in colorectal cancers. Its function as a target for cancer therapy has already been addressed in the nontumoral mucosa in mice, the B-subunit targets spontaneous digestive tumorigenesis. Furthermore, STxB accumulation in the intestinal pathogen-produced Shiga toxin has evolved molecular properties that are of interest in this context. Its nontoxic B-subunit binds to the cellular toxin receptor, glycosphingolipid Gb3, which is highly expressed on human cancers and has recently been reported to be involved in the formation of metastasis in colorectal cancers. Its function as a target for cancer therapy has already been addressed in the nontumoral mucosa in mice, the B-subunit targets spontaneous digestive tumorigenesis. Furthermore, STxB accumulation

Introduction

Cell transformation and oncogenic development are accompanied by changes in the expression and the structure of glycosphingolipids (1). The glycosphingolipid globotriaosyl ceramide (Gb3 or CD77) is expressed on a narrow range of committed B cells and associated B-cell lymphomas (2–7). Ovarian hyperplasias (8), cell suspensions obtained from human breast tumors (7), testicular seminomas (9), colorectal carcinomas (10), and other small intestine tumors of different origins (4) were tested positive for this lipid. Finally, Gb3 was also markedly increased in cell lines derived from human astrocytomas (11). Thus, Gb3 can be considered as a tumor marker, like other glycosphingolipids.

Natural Gb3 ligands exist, among which Shiga toxin and the related verotoxins are the best-studied examples. Shiga toxin is produced by Shigella dysenteriae and the highly related verotoxins (or Shiga-like toxins) by enterohemorrhagic Escherichia coli strains (12). The verotoxins are responsible for pathologic manifestations that can lead to hemolytic uremic syndrome, the leading cause for pediatric renal failure (13). These protein toxins are composed of two subunits, the catalytic A-subunit that inhibits protein biosynthesis by modifying rRNA and the nontoxic homopentameric B-subunit (STxB). STxB binds to 10 to 15 Gb3 molecules (14), likely leading to lipid clustering in membrane microdomains (15), an important event for the intracellular trafficking of the holotoxin (16, 17). In toxin-sensitive cells, Shiga toxin and the nontoxic STxB are transported from the plasma membrane to the endoplasmic reticulum via the early endosome and the Golgi apparatus, a transport pathway termed the retrograde route (refs. 18, 19; for a review, see ref. 20). At the level of the endoplasmic reticulum, the catalytic A-subunit of Shiga toxin uses the cellular retrotranslocation machinery to have access to the cytosol whereas STxB remains in the endoplasmic reticulum/Golgi membrane system (21). In the cytosol, the A-subunit modifies rRNA, leading to an inhibition of protein biosynthesis.

In the light of the described Gb3 expression on human cancer cells, it is tempting to propose the use of its natural ligand, Shiga toxin, for tumor cell delivery purposes. Shiga toxin as a cytotoxic agent has been tested in xenograft tumor models in mice (11, 22–25). Furthermore, it eliminates clonogenic tumor cells in purging applications (26). However, the use of the holotoxin as a therapeutic agent in humans may have limitations because the action of the A-subunit of the toxin is not tumor cell specific.

We have therefore set out to use Gb3-binding STxB as a delivery tool in the absence of the A-subunit. A STxB variant was constructed that allows site-directed chemical coupling (27, 28), preserving the interaction with Gb3 and the intracellular trafficking characteristics of STxB.

In this study, we choose digestive tumors to show the tumor targeting capacity of STxB because they are among the most frequent malignancies in Europe and North America and represent a well-studied paradigm for solid tumor formation. Using classic histology, we show the in vivo accumulation of STxB-delivered contrast agents in tumors of different mouse models for spontaneous digestive tumorigenesis. Furthermore, STxB accumulation

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-0631

4 Manuscript in preparation.
in tumors was observed in anesthetized animals by fibered confocal laser endoscopy after force-feeding and by positron emission tomography (PET) imaging after i.v. injection. We conclude that the naturally evolved properties of STxB, notably the high number of tumor cell binding sites and its trafficking via the retrograde route, can be diverted for tumor cell delivery of exogenous compounds, such as contrast agents for tumor imaging and possibly also therapeutic compounds with preferential effects on cancer cells.

Materials and Methods

Lipid extraction and TLC overlay. Frozen tissue samples of murine origin were weighted and mechanically homogenized in 1 mL of water. Extraction, overlay, and quantification were done as described (16).

Purification of STxB from bacteria and labeling. STxB was purified from bacteria as previously described (29) and dia lyzed against PBS before force-feeding. Coupling to the fluorophores (Amersham Biosciences, Piscataway, NJ) was carried out according to the instructions of the supplier.

Animal models and oral administration of STxB. All experiments involving mice were carried out with the approval of the local authorities. The animals used in the present study were >6 months of age and 25 to 35 g in weight at the time of injection. The mice were maintained under a 12-hour light-dark cycle and fed with standard diet and water ad libitum. A flexible plastic needle (Marquet Genie Biomedical, Bossy Saint Leger, France) was used for force-feeding of anesthetized animals through the esophagus with a single dose of 0.3 to 0.5 mL of a solution of varying STxB concentrations (typically ~1 mg/mL). More than 20 mice were analyzed by immunohistochemistry.

Murine tumor analysis. Intestinal specimens were processed for immunohistochemistry immediately after sacrificing the animal, essentially as described (30). Tumors were identified by standard histologic criteria and using the proliferation marker Ki67, and classified according to WHO guidelines (31). Adenocarcinomas were considered invasive if malignant epithelial cells, arranged in glandular and/or trabecular structures, were found invading at least the submucosa. Antibodies used were monoclonal antibodies (mAb) and polyclonal antibodies specific for STxB (16), mAb anti-villin ID2C3 (32), polyclonal antiserum anti–cholera toxin B (ProGen, Heidelberg, Germany), anti-Ki67 pAb (Novocasta, Newcastle upon Tyne, United Kingdom), anti-CD11b and anti-CD11c coupled to FITC (BD Pharmingen, San Diego, CA), and median Golgi marker mAb CTR433 (M. Bornenis, Paris, France). Secondary antibodies were goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG coupled to Alexa 488 or Cy3 (Jackson Immunoresearch, West Grove, PA). The dye Hoechst 33258 (Sigma, Lyon, France) was used to stain nuclei. For the staining of endogenous Gb3, tissue was fixed for 20 minutes at room temperature with 3% paraformaldehyde, and cryosections were incu bated for 30 min with STxB coupled to the fluorophore Cy3 at a final concentration of 10 μg/mL in PBS containing 0.2% bovine serum albumin. Primary tumor cells were isolated and cultured essentially as described (33).

Confocal laser endoscopy. A commercially available fiber optics device (Cell-viZio from Mauna Kea Technologies, Paris, France) was used. After being on diet overnight, eight animals were force-fed with FITC-coupled STxB and were anesthetized 2 hours later by a mixture of fentanyl, xylazine, and ketamine. A small incision was made in the ventral skin and the peritoneum, and the fiber optics probe was introduced in the lumen of the intestine through a small cut. The fiber optics probe (diameter, 1.65 mm) did not cause any obvious damage to the intestinal mucosa. Dynamic observations were made using 488-nm wavelength laser. Immediately after the endoscopic observation, animals were sacrificed and the tissue was collected, frozen, and processed for immunostaining.

MicroPET imaging studies. The PET imaging studies were done on six transgenic mice, ages 12 or 16 months, after birth on a microPET FOCUS 220 scanner (Concorde Microsystems-CTI). Mice were anesthetized with isoflurane (induction, 3.5%; maintenance, 1-2.5%) and i.v. injected with 400 μCi (14.8 MBq) of 18F-labeled STxB (28) in 100 μL of saline solution. One hour after injection, the mice were reanesthetized and placed in the center of the field of view of the camera, and PET images were acquired during 1 hour. Images were reconstructed using the FORE-OSEM two-dimensional algorithm after correction for 511-keV tissue attenuation by a segmented map of the emission acquisition. Reconstructed images were analyzed using the Asipro VM analysis tool (Concorde Microsystems-CTI). Following the imaging session, animals were sacrificed by i.v. administration of a lethal dose of pentobarbital and a macroscopic inspection of the abdomen was immediately done.

Results

Gb3 expression in mouse models of spontaneous digestive tumorigenesis. Gb3 was recently described to be specifically expressed on human colorectal carcinomas with functions in the formation of metastasis (10). We here analyzed its expression in three genetically defined mouse models of spontaneous digestive tumorigenesis: one that expresses oncogenic K-ras under control of the intestinal epithelium specific villin promoter (referred to as KRAS+/1638N; ref. 30); a second that carries the heterozygous mutated allele Apc−/− (1638SN) of the adenomatous polyposis coli locus (referred to as Apc−/1638SN; ref. 34); and the compound mutant KRAS+/1638N/Apc−/1638SN mouse.5 All three mouse models gave essentially the same results when using neutral glycosphingolipid extraction and TLC to determine the expression levels of Gb3 (Fig. 1A and B). A variable increase in Gb3 levels in tissue blocks of intestinal adenocarcinomas was observed as compared with adjacent normal intestine. To analyze which cells express Gb3, the lipid was detected on cryosections using fluorophore-labeled STxB (Fig. 1C and D). Normal epithelial tissue was found to be Gb3 negative (Fig. 1C). Some occasional cells showed Gb3 labeling (see below for identification). In contrast, tumors were uniformly stained (Fig. 1D), especially the epithelial tumor cells in glandular and trabecular structures.

In summary, these results directly show that Gb3 is expressed by mouse tumor cells of epithelial origin.

Orally administered STxB is able to target mouse adenocarcinoma cells in vivo. To test whether orally administered STxB was able to reach digestive tumors, transgenic mice were sacrificed at variable intervals after force-feeding with fluorophore-labeled STxB-containing solutions. Sections from normal small intestine and tumor regions were prepared and stained for nuclei [Fig. 2; 4′,6-diamidino-2-phenylindole (DAPI), blue] and other markers, as indicated. After 2.5 hours, we found that normal intestinal tissue was overall negative for STxB staining, except for occasional cells interspersed in the epithelial layer (Fig. 2A, red). These STxB-positive cells had the morphologic characteristics of enteroendocrine cells and were recognized by chromogranin A/B (Fig. 2C, green), a marker of these cells. Adenocarcinomas from the periampullar region, duodenum, and jejunum were found to have strongly accumulated STxB (Fig. 2B, red). STxB had clearly entered tumoral cells of epithelial origin that lined trabecular or glandular structures. Furthermore, stromal regions with signs of inflammation sometimes contained STxB-labeled cells (Fig. 2D, red) that were CD11b positive (green). These cells are likely to be infiltrating macrophages but could also be natural killer cells or B-cell subpopulations. The analysis of F-actin staining (red) in normal (Fig. 2E) and tumoral regions (Fig. 2F) revealed the organization of the epithelial layer and indicated that STxB-positive tumor cells

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binding isotherms of STxB to cells depend on cellular Gb3 levels in intracellular distribution of Gb3 vary between normal epithelial cells and tumor cells (e.g., in tumor cells, a higher fraction of Gb3 capacity of STxB, up to 15 Gb3 molecules per pentamer (35), efficient binding. Indeed, because of the multiligand binding normal epithelial cells is below a threshold level required for cancer Research 2006; 66: (14). July 15, 2006 7232 www.aacrjournals.org

A, STxB overlay experiment on neutral glycosphingolipid extracted from the indicated samples (Ctrl, colon adjacent to tumor regions). Glycosphingolipids from HeLa cells were loaded as a positive control, next to standard (Std) Gb3. B, quantifications of overlay experiments, Gb3 expression in normal (white columns) or tumor (gray columns) intestinal tissue samples from transgenic mice. Black columns, averages. C and D, detection of Gb3 on fixed tissue sections with Cy3-labeled STxB (red). Normal jejunum (C) and adenocarcinoma (D) from the same animal. Whereas few cells are positive in normal tissue, tumor sections show strong staining. Blue, nuclear control staining with DAPI. Bar, 50 μm.

Figure 1. Gb3 expression in mouse models of digestive tumorigenesis. (green) were indeed located in cells of epithelial origin. In normal tissue, the STxB-positive cells resemble enteroeendocrine cells interspersed in the epithelial layer. In tumors, the STxB-positive cells were organized in epithelial glandlike structures.

The question arises on why relatively subtle differences in Gb3 levels between adjacent versus tumor regions, as detected by lipid extraction (Fig. 1A and B), translate into substantial differences in STxB accumulation in normal versus tumoral epithelial cells (Fig. 24 and 2B, respectively). We hypothesize that Gb3 expression in normal epithelial cells is below a threshold level required for efficient binding. Indeed, because of the multiligand binding capacity of STxB, up to 15 Gb3 molecules per pentamer (35), binding isotherms of STxB to cells depend on cellular Gb3 levels in a complex nonlinear manner. It is also possible that the intracellular distribution of Gb3 varies between normal epithelial cells and tumor cells (e.g., in tumor cells, a higher fraction of Gb3 might be at the plasma membrane). A further possibility would be a higher turnover of STxB in normal epithelial cells, leading to a lower accumulation at steady state. These different possibilities still need to be tested directly.

Even 24 hours after force-feeding mice with STxB containing solutions, normal intestinal epithelium did not take up STxB (Fig. 2G, red) except for enteroeendocrine cells. In contrast, STxB was still strongly present in tumoral tissue (Fig. 2H, red). No staining was observable in liver or kidney sections (not shown).

To understand the reasons for the observed stable association of STxB with intestinal tumors, we developed primary tumor cell cultures in which intracellular STxB transport was analyzed by immunofluorescence. After 45 minutes of internalization, STxB (Fig. 3A, red) significantly colocalized with the medial Golgi marker CTR433 (Fig. 3B, green; overlay in Fig. 3C), indicating that STxB followed the retrograde route in primary tumor cells from the plasma membrane to the Golgi apparatus, avoiding recycling or degradation. Three days after internalization, STxB was still detectable in punctate structures (Fig. 3D, red) although no obvious colocalization with Golgi structures could be detected (Fig. 3E, green; overlay in Fig. 3F). These structures partially overlapped with the endoplasmic reticulum (not shown). STxB was also detected up to at least 3 days in cultures of epithelial tumor cells obtained from mice that had been force-fed (not shown).

In situ detection of intestinal tumors by confocal laser endoscopy. Endoscopy of the colon is the clinical standard method for detection of neoplasia. We used a commercially available fibered confocal optics device (Cell-viZio; Fig. 4A) to detect in situ tumors labeled with fluorescent STxB. For this, the chemical cross-linking variant of STxB, STxB/Cys (27), was coupled to fluorescein. Using mass spectroscopy, it was shown that all B-chains of the STxB/Cys pentamer were fluorophore modified. Untreated mice or mice that were force-fed with fluorescent STxB were anesthesized and the fiber optics cable was introduced by a small incision into the lumen of the gut. In untreated transgenic and wild-type animals, no specific signal was detected (not shown). After force-feeding with fluorescein-coupled STxB, isolated single cells were visible in normal tissue of wild-type animals (Fig. 4B, image a; Supplementary data, Film 1). According to immunocytochemistry done on the same region (Fig. 4C, image a'), these cells most likely corresponded to enteroeendocrine cells. In force-fed transgenic animals, we observed a strong STxB accumulation in different parts of tumoral lesions (Fig. 4B, image b; Supplementary data, Film 2). This accumulation was confirmed by immunocytochemistry to be STxB specific (Fig. 4C, image b').

These experiments show the feasibility of endoscopic detection of digestive tumors using STxB-based fluorescence colonoscopy in living mice.

Systemic injection for detection of adenocarcinomas of the gut by PET imaging. In another series of experiments, we tested...
radioactivity was found in the urinary tract as shown by the high levels of radioactivity in the bladder and kidneys (Fig. 5A-C). Uptake was also observed in the spleen, lungs, and, to a lesser extent, in the liver. In wild-type mice, the abdominal region was devoid of labeling (Figs. 5A and 6). In contrast, coronal sections of microPET images (15-minute frame duration) of a 16-month-old tumor-bearing APC mouse at 60 minutes after i.v. injection of \(^{18}\text{F}\)-labeled STxB showed a high level of STxB uptake at two sites of the digestive tract (Fig. 5B and C; arrows; Supplementary data, Film 3 and Fig. 6B and C). One of these sites (Fig. 5B, red arrow) was already observed in the same area at 12 months of age, 4 months before this PET examination (not shown). The continued capacity of STxB to target the tumors after multiple injections suggests that even if an immune response was raised against STxB, it did not prevent STxB from reaching the tumors. The mouse was then sacrificed and the presence of tumors at the expected sites, the periampullar area (red arrow) and one in the lower right abdomen (green arrow), was confirmed visually (Fig. 5D). GB3 extraction and overlay allowed to ascertain that both tumors indeed expressed GB3.

In the case of another animal, the digestive tube was entirely removed and imaged directly. Even on the opened and washed digestive tract, labeling could be detected at selected sites (Fig. 5E), which corresponded to visually detectable lesions (Fig. 5F).

Similar analysis on four more animals confirmed all of these observations and showed that tumor detection by \(^{18}\text{F}\)-labeled STxB PET was robust (data not shown). The mean ratio of \(^{18}\text{F}\)-labeled STxB uptake in the abdominal tumors reached values of 3 versus the adjacent intestinal tract and 1.7 versus the liver.

In conclusion, STxB-based delivery of fluorescent or PET contrast agents indicates that STxB has the potential to be used for molecular imaging of digestive tumors.

**Discussion**

In this study, we have developed a novel tumor delivery approach that exploits naturally evolved characteristics of STxB. Using mouse models of spontaneous intestinal adenomatosis, it was found that on force-feeding, STxB accumulates in these tumors and remains associated with them for several days. We show that this remarkable fact is due to the targeting of STxB into a recently described intracellular transport pathway, the retrograde route. This approach was used for generating contrast *in vivo* at the cellular scale. Using a novel fiber optics confocal microscope that has already been adopted to endoscopic settings in the clinics, we show that colorectal neoplasia can be detected after force-feeding mice with STxB-coupled fluorescent probes. PET experiments show that intestinal tumors in mice are also accessible to STxB if applied via the systemic route.

The need to achieve optimal tumor retention is one of the critical points about targeted delivery approaches (36). In this respect, one of the main advantages of STxB as a vector resides in its intracellular targeting via the retrograde route. Indeed, after its uptake by endocytosis, STxB is neither degraded nor recycled to the plasma membrane (for a review, see ref. 20). Rather, the protein leaves endosomes to reach other compartments in the cell, such as the Golgi apparatus and the endoplasmic reticulum. In this study, we could show that these observations are not only true in cancer cell lines but also in primary tumor cells. This retrograde transport-mediated stable association with tumor cells could be of great value for tumor imaging because

Whether the digestive tumors of the above-mentioned mouse models could be reached by STxB-coupled contrast agents after i.v. administration. To address this question, we used PET imaging. STxB was chemically coupled to a recently described \(^{18}\text{F}\)-fluoropyridine-based maleimide reagent (28). Using mass spectroscopy, it was observed that all B-chains of the STxB/Cys pentamer were modified, and the functionality of the coupling product was confirmed by cellular trafficking assays.

Mice were imaged between 1 and 2 hours after retro-orbital injection of \(^{18}\text{F}\)-fluoropyridine-STxB. In all cases, the bulk of whether the digestive tumors of the above-mentioned mouse models could be reached by STxB-coupled contrast agents after i.v. administration. To address this question, we used PET imaging. STxB was chemically coupled to a recently described \(^{18}\text{F}\)-fluoropyridine-based maleimide reagent (28). Using mass spectroscopy, it was observed that all B-chains of the STxB/Cys pentamer were modified, and the functionality of the coupling product was confirmed by cellular trafficking assays.

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it would allow contrast agents to be cleared from nontumor tissues before image acquisition is started. Furthermore, in some cell types, such as monocytes and macrophages, Shiga toxin has no effect on protein biosynthesis (37, 38) due to failure to reach the retrograde route (16). This difference in trafficking between cell types offers interesting opportunities for the development of innovative delivery strategies.

As a product of enteropathogenic bacteria (12), STxB has naturally evolved to withstand the degrading environment of the intestinal lumen (extreme pH in the gastrointestinal tract, high protease activity) and to cross the intestinal barrier. Furthermore, STxB is a low immunogenic protein (see ref. 20 for a review). These data indicate that STxB could be safely used as a delivery tool.

Prognosis of colorectal carcinoma is mainly based on local invasion and distal dissemination, with a 5-year survival of <10% when metastases are present. The Donowitz group has recently described the expression of Gb3 by metastatic human colorectal carcinomas (10). As a target, Gb3 may thus allow detection of metastasis by noninvasive imaging approaches, such as STxB-based PET imaging, as described in this study. Indeed, the PET experiments showed the capacity of 18F-labeled STxB to accumulate in tumors after systemic administration. Cancer staging with PET is currently done with 18F-fluorodeoxyglucose, a biomarker of glucose consumption, which produces contrast because it accumulates more readily in highly metabolically active tumoral cells than in normal tissue. STxB may offer an interesting alternative to 18F-fluorodeoxyglucose in cases where the uptake of this tracer by tumors is low or produces little contrast or false positive because of the presence of metabolically active nontumoral cells, and help circumvent some current limitations of 18F-fluorodeoxyglucose in colorectal and other cancers.

Another potential application of our findings concerns the use of STxB as a delivery tool for contrast agents for confocal laser endoscopy. Colonoscopy is, to date, the most powerful method to...
investigate polyp, adenoma, or tumor growth in the human intestine. This is also due to the possibility to obtain biopsy samples from regions in which abnormalities can be detected optically. In some situations, however, tumor localizations are not optically detected, requiring systematic biopsies. This is the case of mantle cell lymphoma in which colorectal microscopic involvement could be found in >88% of cases (39). Confocal laser endoscopy would allow detection of Gb3-positive sites combined with analysis of tissue architecture (40) to permit target directed selection of biopsies. More generally, our method may permit to improve the performance status of colonoscopy whenever detection of flat or intraepithelial lesions requires histologic analysis to secure the diagnosis. Indeed, taking too many biopsy samples or doing biopsy instead of resection of neoplastic tissue can result in unnecessary risks, such as bleeding. In addition to polyps in the large bowel, human small intestine tumors of different origins (lymphomas, carcinomas, and leiomyosarcomas) are Gb3 positive.6 Confocal laser endoscopy does not constitute an appropriate method of detection for these malignancies but STxB could be used in other applications, such as microcamera detection of STxB-coupled contrast agents or PET detection of 18F-labeled STxB.

STxB seems to have a large spectrum of applications, such as diagnostic delivery of contrast agents for confocal laser endoscopy, PET, resonance magnetic imaging, or isotope scanning. The Shiga delivery technology described in this study opens new avenues for the development of innovative approaches to molecular tumor imaging and, possibly, targeted tumor therapy.

Acknowledgments

Received 2/17/2006; revised 5/5/2006; accepted 5/23/2006.

Grant support: Centre National de la Recherche Scientifique/CEA (Imagerie du petit Animal); Ministry of Research (ACI Biologie du Développement et Physiologie Intégrative); Fondation de France; Association pour la Recherche sur le Cancer grants 3105, 4803, and 5177; the EMIL European network; Cancéropôle Ile-de-France; Human Frontier Science Program (D. Vignjevic); Fondation pour la Recherche Médicale (G. Bousquet); Ligue Nationale contre le Cancer and Fondation pour la Recherche Médicale (T. Falguieres); and Deutsche Forschungsgemeinschaft and Kommission für Klinische Forschung (K-P. Janssen).

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We thank Mauna Kea Technologies (http://www.maunakeatech.com) for support, especially Charlotte Casé and Philippe Crouzet from Estium-Concept for drawing, and Sandrine Meunier, Vincent Border, Carmen Marthen, Bertrand Kuhnast, and Françoise Hinnen for technical assistance.

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Figure 5. MicroPET coronal images of wild-type and transgenic mice 60 minutes after i.v. injection of 14.8 MBq of [18F]STxB. A, in the wild-type mouse, no labeling was detected in the abdominal region. B and C, on two coronal sections of a transgenic animal (mouse 548), strong labeling was observed in the top (B, red arrow) and bottom (C, green arrow) parts of the digestive tract. For three-dimensional reconstructions of images obtained from mice shown in (A-C), refer to Fig. 6 and Supplementary data, Film 3. D, the mouse was sacrificed and tumors in the peripampullar area (red arrow) and the lower abdominal area (green arrow) could be detected in the opened abdomen. E and F, the intestine extracted postmortem from another mouse immediately after [18F]STxB PET showed the optical presence of tumors (F) at the same sites where the radioactivity uptake was detected (E). Secondary sites of accumulation: Bl, bladder; Lu, lung; Li, liver; Sp, spleen.

Figure 6. Views from different angles of projection images reconstructed from whole-body data sets of PET acquisitions. These views depict the three-dimensional distribution of the tracer in terms of radioactivity concentrations in mice organs 2 hours after injection of a tracer dose of 18F-labeled STxB. A, control mouse from Fig. 5A seen from its ventral side. B, tumoral mouse from Fig. 5B and C and Film 3 seen from its ventral side. Green arrow, tumor in the lower abdomen. C, same mouse as in (B) and in Fig. 5B and C and Film 3, seen from its right side. Green arrow, the same lower abdomen tumor shown in (B); red arrow, a peripampullar tumor.

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6 Manuscript in preparation.
References


Cancer Res 2006; 66: (14). July 15, 2006 7236 www.aacrjournals.org

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