Positherapy: Targeted Nuclear Therapy of Breast Cancer with $^{18}$F-2-Deoxy-2-Fluoro-$\beta$-Glucose

Renee M. Moadel,1 Richard H. Weldon,2 Ellen B. Katz,2 Ping Lu,1 Joseph Mani,1 Mark Stahl,3 M. Donald Blaufox,1 Richard G. Pestell,1 Maureen J. Charron,2 and Ekaterina Dadachova

1Department of Nuclear Medicine, Albert Einstein College of Medicine of Yeshiva University; Departments of 2Biochemistry and 3Cell Biology, Albert Einstein College of Medicine, Bronx, New York; and Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, District of Columbia

Abstract
Breast cancer remains a major cause of cancer death in women in the United States. Novel therapies are needed for patients when standard treatments are ineffective. We have recently shown on a cellular level the therapeutic potential of positrons in malignancy. Here, we report for the first time positron therapy with $^{18}$F-2-deoxy-2-fluoro-$\beta$-glucose ($^{18}$F-FDG) in a breast cancer animal model to affect tumor growth rate and survival (positherapy). We used xenografted mammary tumors in nude mice using Notch mammary cancer cells which also express ras oncogene. Notch xenografted tumors actively took up $^{18}$F-FDG with a tumor to normal tissue ratio of 3.24. Tumor-bearing mice were treated with 2.5 mCi $^{18}$F-FDG, which is equivalent to the physiological human maximum tolerated dose. Positherapy resulted in both significant prolongation of survival and decrease in tumor growth rate in comparison with nontreated controls. Immunoblot of Notch tumors showed the presence of glucose transporters (GLUT) 1, 4, and 8. Substantial differences between GLUT1, GLUT4, and GLUT8 were observed in their distribution within the tumor mass. Whereas GLUT4 and GLUT8 were distributed relatively homogeneously throughout the tumor, GLUT1 was confined to necrotic areas. Immunofluorescence double labeling was used to determine cellular localization of GLUTs. GLUT1 was expressed mostly at the cell membrane. GLUT4 and GLUT8 were mostly localized to cytoplasmic compartments with some GLUT4 expressed at or near the cell membrane in close proximity to GLUT1. Thus, GLUT1 was likely responsible for the $^{18}$F-FDG uptake by tumor cells with some possible contribution from GLUT4. These results are important for the development of positherapy with $^{18}$F-FDG for refractory metastatic breast and other cancers. (Cancer Res 2005; 65(3): 698-702)

Introduction
Breast cancer remains a major cause of cancer death in women in the United States. Novel therapies are needed for patients when surgery, chemotherapy, hormonal treatment, and external beam radiation are not effective. $^{18}$F-2-deoxy-2-fluoro-$\beta$-glucose ($^{18}$F-FDG) is widely used in positron emission tomography (PET) for the evaluation of patients with tumors. Recently, $^{18}$F-FDG-PET has become a well-established modality in the evaluation of breast cancer (1). $^{18}$F-FDG, an analogue of glucose, is taken up by cells and phosphorylated by hexokinase to $^{18}$F-FDG-6-phosphate. Because $^{18}$F-FDG-6-phosphate is not a substrate for further glycolysis, and is not readily dephosphorylated, it remains trapped in the cell (2). $^{18}$F emits positrons with an average energy of 0.250 MeV and 96% abundance. Positrons (positively charged electrons) lose their kinetic energy and damage tissue in the same manner as electrons. Theoretically, positrons should kill cancer cells as electrons.

We have recently shown on a cellular level the therapeutic potential of positrons in malignancy, showing apoptosis and necrosis in mammary cancer cells caused by $^{18}$F-FDG (3). Here, we investigate for the first time the potential of positron therapy with $^{18}$F-FDG in a breast cancer animal model to affect tumor growth rate and survival (positherapy). For induction of tumors in animals, we have chosen mouse mammary gland cancer cell line 5505 which expresses an activated form of Notch4 (4). The Notch gene influences cell differentiation and cell fate during development and needs to partner with an oncoprotein, such as ras, to cause cancer, and the relevance of Notch and ras in human breast cancer has been shown (5). The transport of interstitial glucose into cells is mediated by a family of homologous glucose transport proteins (GLUT), which differ in their tissue distribution and physiological properties (6). As the ras oncogene increases cellular glucose uptake (7) and translocation of GLUT to the cell membrane (8), we hypothesize that Notch mammary cancer cell line would have increased transport of $^{18}$F-FDG into the cells to enhance the effect of positron therapy.

Materials and Methods
Cell Line. Mouse mammary gland cell line 5505 has been derived from mammary tumors arising in transgenic mice which express an activated form of Notch4 from the mouse mammary tumor virus long terminal repeat promoter/enhancer (4). These cells (further referred to as Notch cells) also express the ras oncogene (4). Notch cells were cultured in DMEM supplemented with 10% FCS, 10% NCTC-109 medium, 1% nonessential amino acids, 4.5 mg/L $\beta$-glucose, and 1% penicillin-streptomycin.

Animal Model. Six- to eight-week-old female nude mice were injected s.c. with $10^5$ Notch cells on their right flank. One week after implantation the tumors reached 0.5 to 0.7 cm in diameter and imaging and therapy with $^{18}$F-FDG was initiated.

PET-Computed Tomography Imaging of Mice with $^{18}$F-FDG. Notch tumor-bearing animals were fasted for 4 hours before being anesthetized with a mixture of 125 mg/kg ketamine and 10 mg/kg xylazine. An i.p. injection of 0.5 mCi $^{18}$F-FDG (Eastern Isotopes, Sterling, VA) was administered. Mice rested while anesthetized for an uptake period of 1 hour. A PET-Computed Tomography (CT) scan was done on a PET-CT scanner (Gemini, Philips, The Netherlands). A 6-minute emission scan followed by a CT scan (15 mA) were obtained. Regions of interest (7 mm diameter) were drawn around the tumor and normal muscle. The tumor-to-normal muscle ratio (TNR) was calculated according to the formula: TNR = average counts per pixel in tumor / average counts per pixel in normal muscle.
The estimated dose delivered to the tumor was calculated using the formula: \( D = \frac{1.44}{C} \times \frac{A_0}{T_e} \times S \), where \( A_0 \) is the initial activity in the tumor, \( T_e \) is the effective half-life of the radiopharmaceutical, and \( S \) is the absorbed dose per unit of cumulated activity. The uptake of \( ^{18}\text{F}-\text{FDG} \) in the tumors was quantified from PET-CT images; \( A_0 \) was calculated by multiplying tumor percent uptake by administered activity; \( T_e \) was assumed to be equal to \( ^{18}\text{F} \) physical half-life; and \( S \) for \( ^{18}\text{F}-\text{FDG} \) for a tumor with a mass of 1 g was obtained from MIRDOS 3 program.

**Treatment of Mice with \( ^{18}\text{F}-\text{FDG} \).** Ten Notch tumor–bearing mice with tumor sizes of 0.5 to 0.7 cm in diameter were fasted for 4 hours and treated i.p. with 2.5 mCi \( ^{18}\text{F}-\text{FDG} \). The mice rested while anesthetized for an uptake period of 1 hour. Ten tumor-bearing control animals did not receive treatment. Animals were monitored for vital signs for 1 month. Tumors were measured every 3 days with calipers in three perpendicular planes. The growth rate for tumors (cm\(^3\))/day was calculated using the formula: (initial tumor volume / tumor volume at day \( n \) post-treatment) / number of days. Kaplan-Meier and log-rank statistics were used to compare survival of the mice in therapy and control groups. The tumor growth rates were compared using the Mann-Whitney test. SPSS 11.5 software was used for statistical calculations.

**Immunoblot Analysis of GLUT1, 2, 4, and 8.** Three nude mice bearing Notch xenografted tumors were sacrificed 1 week post-implantation to match the time of \( ^{18}\text{F}-\text{FDG} \) therapy. Rabbit polyclonal GLUT1, GLUT2 (Alpha Diagnostic International, San Antonio, TX), GLUT4 (9), and GLUT8 (10) isoform-specific antibodies were used for detection. Tumor tissues were prepared and SDS-PAGE carried out as in ref 10. Primary antibodies were diluted 1:2,000 in 5% bovine serum albumin in TBS buffer, 0.1% Tween 20.

**Immunofluorescence of GLUT1, 4, and 8.** Tumors sections (3 μm) were prepared as in ref. 10. Sections were consecutively incubated: 15 minutes in PBST (PBS with 0.3% Triton X-100); 30 minutes in blocking buffer (PBST, 5% normal goat serum, 0.2% bovine serum albumin); 1 hour in rabbit polyclonal antibodies specific to GLUT1 (1:100) or GLUT4 (1:50) or affinity purified GLUT8 (1:2 μg/mL at 1:100) in blocking buffer; 15 minutes in PBST; 1 hour in Cy3-conjugated anti-rabbit IgG diluted (1:1000) in blocking buffer; 15 minutes in PBST; 1 hour with GLUT specific antibody conjugated to Alexa Fluor 488 (Zenon Rabbit IgG Labeling Kit, Molecular Probes, Eugene, OR); 30 minutes in PBST; 15 minutes in 4% paraformaldehyde in PBS and mounted using anti-fade media (Molecular Probes). Peptide competition controls were done by pre-incubating GLUT specific antibodies with 200-fold molar excess of corresponding C-terminal peptide for 30 minutes before application. Other negative controls included substituting GLUT specific antibodies for rabbit IgG (2 μg/mL) and incubation with Cy3 secondary antibody only.

**Results**

**PET-CT Imaging of Notch Tumor–Bearing Mice with \( ^{18}\text{F}-\text{FDG} \).** A coronal PET-CT image of a nude mouse xenografted with Notch mammary cancer cells is presented in Fig. 1A and B. The slice (2 mm thickness) with the highest activity in the tumor was chosen. The TNR for the Notch tumors was 3.24 ± 0.20.

**Figure 1.** 

- **A.** \( ^{18}\text{F}-\text{FDG} \) PET-CT and \( ^{18}\text{F}-\text{FDG} \) therapy of nude mice bearing xenografted Notch tumors: (A) coronal \( ^{18}\text{F}-\text{FDG} \)-PET image of a mouse with a tumor in its flank acquired 1 hour after i.p. injection of \( ^{18}\text{F}-\text{FDG} \). The slice (2 mm thickness) with the highest activity in the tumor was chosen. The mouse is lying on its back with its head pointing up. The red color on the image corresponds to the highest radioactivity uptake. As \( ^{18}\text{F}-\text{FDG} \) is normally excreted in the urine, bladder activity is visible. (B) CT image of the same mouse. The tumor is visualized as soft tissue fullness as compared with the normal contralateral flank. Tumor is marked with an arrow on the PET and CT images. (C) Kaplan-Meier survival curves of Notch-tumor bearing mice treated with 2.5 mCi \( ^{18}\text{F}-\text{FDG} \) in comparison with nontreated mice. (D) H&E-stained section of Notch tumor showing tumor vascularity. Arrows, blood vessels. (E) Notch tumor growth in control (\( n = 2 \), as only two mice lived long enough to have tumor growth measured) and in \( ^{18}\text{F}-\text{FDG} \)-treated animals (\( n = 10 \)). The median tumor growth rates of the control and therapy groups were 1.92 and 0.92 cm\(^3\)/day, respectively (\( P = 0.031 \), Mann-Whitney).
Treatment of Notch Tumor–Bearing Mice with 18F-FDG. The Kaplan-Meier survival curves of Notch-tumor-bearing mice treated with 2.5 mCi 18F-FDG in comparison with nontreated mice are presented in Fig. 1C. Whereas control mice (n = 10) died within 1 week post-treatment with a median (95% CI) time to death of 2 (1–3) days, mice treated with 18F-FDG (n = 10) survived significantly longer with a median time to death of 17 (8–26) days and for periods of up to 30 days (P < 0.001). 18F-FDG significantly slowed tumor growth, and the median tumor growth rates of the control and therapy groups were 1.92 and 0.92 cm3/d during the first 6 days, respectively (P = 0.031; Fig. 1D). The dose delivered by 18F-FDG to the tumor was calculated to be 330 ± 30 cGy. Notch tumors generated hemangiomias, and vascularity of the tumor is seen in the H&E-stained section in Fig. 1E.

Immunoblot and Immunofluorescence of Notch Tumors. Notch-expressing tumors from three mice were screened for the expression of GLUT1, 2, 4, and 8 using immunoblot analysis (Fig. 2). The results show the presence of GLUT1, 4, and 8, however, GLUT2 was undetectable.

Immunofluorescence double labeling was used to assess the distribution of GLUT1, 4, and 8 within the tumor mass as well as within the cell (Fig. 3). Immunofluorescence staining for GLUT1 (A and D) clearly showed a heterogeneous pattern of expression within the tissue with regions of high GLUT1 expression adjacent to areas where GLUT1 was almost absent. In contrast, both GLUT4 (E and G) and GLUT8 (B and H) displayed a more homogenous pattern of staining throughout the tumor mass. In regard to the cellular localization, GLUT1 was expressed mostly at the surface of the cell with relatively little cytoplasmic expression. However, both GLUT4 and GLUT8 were visible throughout the cytoplasm with no obvious abundance at the surface of the cell. This difference was exemplified when images were merged to show expression of two GLUTs simultaneously (C, F, I, and J). When GLUT1 and GLUT8 were combined (C and J) there was almost no overlap of immunostaining, suggesting that GLUT1 was almost exclusively localized at the plasma membrane and GLUT8 in the cytoplasm. When GLUT1 was merged with GLUT4 (F and K), the separation was not as distinct, suggesting that some GLUT4 was expressed at or near the plasma membrane in proximity to GLUT1. Finally, when GLUT4 was combined with GLUT8 (I and L), the cytoplasm of most cells appeared yellow, indicating a close cytoplasmic proximity of these two transporters. Negative control experiments resulted in the disappearance of signal for GLUT1, GLUT4, and GLUT8 (data not shown).

To determine if the expression of GLUT1 was localized to the necrotic areas in Notch tumors, serial sections were stained with H&E and compared with GLUT1 and GLUT8 double immunostained sections (Fig. 3M and N). GLUT1 expression seemed to be associated with the areas of focal necrosis.

Discussion

Metabolic trapping of 18F-FDG is an attractive mechanism to deliver radioactivity to tumors as neoplastic cells have an enhanced rate of glucose utilization (11). With a physical half-life of almost 2 hours, 18F emits energetic positrons with high abundance (96%) and a path length in tissue of ~0.1 to 0.2 cm. These qualities make 18F-FDG a candidate for investigation as an agent for the treatment of breast and other cancers. Previously, we described apoptosis and necrosis of cells in mammary tumors in transgenic mice in response to 18F-FDG (3). Here we report the first use of positrons (positherapy) delivered by 18F-FDG definitively affecting tumor growth rate and survival in animals with mammary tumors.

We used xenografted mammary tumors in nude mice using Notch mammary cancer cells, which also express the ras oncogene (4). This cell line was chosen due to the relevance of the Notch and ras oncoproteins to human breast cancer (5). We hypothesized that this aggressive tumor cell line will have high metabolic rate and, subsequently, high expression of GLUT for transport of 18F-FDG into these cells. Imaging experiments in mice bearing Notch xenografted tumors showed that xenografts actively took up 18F-FDG with a TNR of 3.24. Because of partial volume effects when imaging a 5 to 7 mm tumor in a small animal with a full-sized PET scanner (resolution of 4.5 mm) the actual TNR could be higher. Encouraged by preferential localization of 18F-FDG in Notch tumors, we treated tumor-bearing mice with 2.5 mCi 18F-FDG, which is equivalent to the physiological human maximum tolerated dose (3). In addition, this dose was not radiotoxic in healthy BALB/c mice (3). Positherapy resulted in both significant prolongation of survival and decrease of tumor growth rate in comparison with nontreated controls (P < 0.05). These results are important as they show therapeutic potential of positherapy with 18F-FDG in an aggressive fast-growing tumor.

It is important to establish the GLUT isoforms responsible for 18F-FDG uptake into the Notch tumors of which GLUT profile has not been investigated previously. The immunoblot analysis showed the presence of GLUT1, 4, and 8, and the absence of GLUT2. Whereas the expression of GLUT1, 2, and 4 has been documented in breast cancer (12–14), this is the first report, to the best of our knowledge, of expression of the GLUT8 isoform, an insulin-regulated transporter (15), in breast cancer.

The distribution of GLUT transporters within the tumor mass determines the radiation dose to portions of the tumor. Substantial differences in the localization of GLUT within the tumor mass were observed. Whereas GLUT4 and GLUT8 were distributed relatively homogeneously throughout the tumor, GLUT1 was confined to necrotic areas. The latter observation is consistent with the reported correlation between tumor necrosis and GLUT1 expression in breast cancer (16). It is known that hypoxic tumors are resistant to radiation therapy, thus GLUT1 location in necrotic/hypoxic regions of tumors may enhance the 18F-FDG cell killing effect in regions of hypoxia.

Cell membrane localization of GLUTs is crucial for uptake of 18F-FDG into the tumor cells to enable positherapy. To determine
Figure 3. Immunofluorescent double labeling of GLUT1, GLUT4, and GLUT8 in Notch tumor at two different magnifications. (A) GLUT1 (red); (B) GLUT8 (green); (C) merged images (A and B); (D) GLUT1 (red); (E) GLUT4 (green); (F) merged images (D and E); (G) GLUT4 (red); (H) GLUT8 (green); (I) merged images (H and I); (J) GLUT1 (red) with GLUT8 (green); (K) GLUT1 (red) with GLUT4 (green); (L) GLUT4 (red) with GLUT8 (green); (M) serial section of the tumor stained with H&E. Circle, viable malignant cells surrounding blood vessels; Arrow, area of malignant cells with central necrosis; (N) immunofluorescence detection of GLUT1 (red) and GLUT8 (green) compared with an H&E-stained section. Magnification: (A-I) ×300, (J-L) ×750, (M-N) ×200.
cellular localization of GLUTs, immunofluorescence double labeling was used. GLUT1 was expressed mostly at the surface of the cell. In contrast, GLUT4 and GLUT8 were mostly localized to cytoplasmic compartments with some GLUT4 expressed at or near the plasma membrane in close proximity to GLUT1. Thus, it is likely that GLUT1 is largely responsible for the $^{18}$F-FDG uptake by the tumor cells by virtue of its localization at the cell surface with some possible contribution from GLUT4. As GLUT8 was seen only intracellularly, it does not seem that Notch and ras promoted translocation of GLUT8 to the cell membrane in these tumors. However, GLUT8 may nevertheless be responsible for intracellular shunting of glucose required for the high metabolic rate of tumor cells.

Whereas GLUT1 and possibly GLUT4 seem to be largely responsible for transport of cytolical $^{18}$F-FDG in necrotic regions of tumor, the “cross-fire” effect, when positrons emitted in necrotic regions damage tissue 0.1 to 0.2 cm away, may be responsible for cell death in nonnecrotic regions. In necrotic parts of the tumor, the cells most likely will still be vulnerable to the particulate radiation of $^{18}$F. In fact, radiolabeled small molecules that localize in hypoxic areas such as $^{64}$Cu-ATSM have been proposed as an alternative to radiation therapy for treatment of hypoxic tumors (17). The dose to the tumor was calculated to be 330 ± 50 cGy, which shows that in radionuclide therapy the doses which are suboptimal from the standpoint of external beam therapy can still be effective. Cell death in nonnecrotic regions may also occur through the “bystander effect”—death of nonirradiated cells via cell-to-cell communication (18). It might be possible that alternative GLUTs expressed in breast cancer, such as GLUT5 (13) and recently discovered GLUT12 (19), may play a role in $^{18}$F-FDG uptake in nonnecrotic regions of the tumor.

GLUT1 and GLUT4 are up-regulated by estrogen and progesterone (14). Breast cancer relevant oncogenes such as ras, c-erb-B2, and src increase expression of GLUT and glucose metabolism (7, 19). Nonradioactive 2-deoxy-D-glucose, administered in pharmacologically significant doses, up-regulated GLUT1 expression and glucose uptake and induced apoptosis and cell death in breast cancer cells (20). Thus, hormonal, genetic, and 2-deoxy-D-glucose interventions can be used to increase glucose metabolism in breast cancer cells and to potentiate the therapeutic effects of positherapy with $^{18}$F-FDG.

In conclusion, we show for the first time that positherapy delivered by $^{18}$F-FDG to Notch mammary tumors in mice both significantly slowed tumor growth and caused prolongation of survival in treated animals in comparison with nontreated controls. These results are important for the development of positherapy with $^{18}$F-FDG for refractory metastatic breast cancer and beyond.

Acknowledgments

Received 9/20/2004; revised 11/15/2004; accepted 11/22/2004.

Grant support: NIH grants DK47425 and HL58119 and ADA grant (M.J. Charron).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Positherapy: Targeted Nuclear Therapy of Breast Cancer with $^{18}$F-2-Deoxy-2-Fluoro-d-Glucose


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/65/3/698

Cited articles  This article cites 20 articles, 9 of which you can access for free at: http://cancerres.aacrjournals.org/content/65/3/698.full#ref-list-1

Citing articles  This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/65/3/698.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.