Evidence of ^{18}F-FCH Uptake in Human T98G Glioblastoma Cells

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Abstract. *Aim: Tumor and chemo/radiotherapy-damaged brain tissues are hardly distinguishable by conventional morphological imaging. ^{18}F-FCH was compared against ^{18}F-FDG in the T98G glioblastoma cell line with regard to their radiopharmaceutical uptake, in order to test its diagnostic power on brain tumor lesions. Materials and Methods: Equimolar amounts of ^{18}F-FCH and ^{18}F-FDG were added to human glioblastoma T98G cells and human dermal fibroblasts for 20, 40, 60, 90 and 120 min of incubation. Radiopharmaceutical uptake was expressed as a percentage of the administered dose. Cold choline was used for binding competition experiments. Results: In T98G cells ^{18}F-FCH was taken-up in higher amounts than 18F-FDG after 60 min. In fibroblasts, uptake was lower than 1% for both radiopharmaceuticals. Cold choline reduced the uptake of FCH to 1% similarly to fibroblasts. Conclusion: Our results prove the efficacy of ^{18}F-FCH as a promising tracer, better than ^{18}F-FDG in establishing the tumor-to-background ratio in brain tumors.*

Malignant gliomas and metastatic brain tumors are the most common forms of brain cancer. Magnetic resonance imaging (MRI) and computed tomography (CT) are very sensitive structural diagnostic tools capable of determining the size and location of brain lesions. However, one documented drawback is their low specificity in identifying post-treatment alterations, such as central necrotic areas and peri-tumoral edema and aspecific signs of surgery, chemotherapy and radiotherapy-induced damage (1). This drawback, together with the need for greater accuracy in assessing brain tumors, has driven medical research towards innovative complementary imaging methods, which provide both a morphological and functional evaluation (2). ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is the most widely used positron emission tomography (PET) radiopharmaceutical for cancer localization, but it has a low sensitivity and specificity for brain lesions due to physiological grey matter uptake. This results in a low tumor-to-background signal ratio and makes it difficult to detect tumors displaying poorer glucose metabolism, as in the case of low-grade gliomas, or to differentiate tumoral lesions from healthy tissue or brain tumor recurrence from therapy-induced necrosis (3). To overcome these limitations, along with the use of several labelled amino acid analogues, Choline (CH)-based radiotracers have already been recognised as being able to depict brain tumors (4) and being hallmarks of malignancy in many cancers (5, 6). Moreover, ^{18}F-Fluorocholine (^{18}F-FCH) is more practical with its advantage of labelling with the longer physical half-life of ^{18}F and is not taken-up by healthy cortex as with ^{18}F-FDG (7), thus providing an effective tool to diagnose brain tumors (3). Choline (CH) is a precursor of phospholipids and as such is used to synthetize cell membranes. Choline is transported into cells by specific mechanisms and then phosphorylated by choline kinase (CK) and further metabolized into phosphatidylcholine that is the major phospholipidic component. Rapidly proliferating tumors demonstrate increased membranes and fatty acid requirements and thus malignant tumors have high levels of CK activity, associated with an increase in the CH cellular specific transport.

The objective of the present *in vitro* study was to compare the ^{18}F-FCH *versus* ^{18}F-FDG in the T98G glioblastoma cell line and fibroblast cells, with respect to their uptake, in order to evaluate the radiopharmaceuticals differential diagnostic power between tumor lesions and healthy tissues.

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Materials and Methods

Cell lines. Human glioblastoma T98G cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured in Eagle's Minimun Essential medium (EMEM, Euroclone SpA, MI, Italy) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 0,01% sodium pyruvate at 37˚C in a humidified atmosphere of 5% CO_2 in air. Human dermal fibroblasts were used as non-pathological control cell type. Primary cultures of human dermal fibroblasts were derived from biopsies taken-up from healthy donors with informed consent. Primary cultures of fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone SpA, MI, Italy) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 2 mM glutamine (Euroclone SpA, MI, Italy) at 37˚C in a humidified atmosphere of 5% CO_2 in air. Stock cultures of both cell lines were maintained in exponential growth as monolayers in 25 cm^2 Corning plastic tissue-culture flasks (Sigma-Aldrich, St Louis, MO, USA).

Radioactive tracer incubation. ^{18}F-FCH was obtained from IASON (Graz-Seiersberg, Austria) and ^{18}F-FDG from IBA Molecular (Monza, Italy). Cells, seeded at a density of 2×10^5 cells per flask when radioactive tracers were administered, grew adherent to the plastic surface at 37˚C in 5% CO_2 in complete medium. Radioactive tracer experiments were performed 20-22 h post-seeding in order to use the cells in the exponential phase of growth. Medium was renewed before perform studies. Cells were incubated at 37˚C with 100 kBq (100 μl) equimolar amounts of ^{18}F-FCH or ^{18}F-FDG added in 2 mL of medium in each flask for different incubation time (20, 40, 60, 90 and 120 min) under 5% CO_2 conditions. Radiotracer incubation was done in complete medium for experiments with 18F-FCH and in PBS supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 2 mM glutamine for experiments with 18F-FDG in order to avoid the competition binding of glucose (1 g/l in the medium) and FDG. Control samples were treated exactly the same as other samples, but they were incubated with 100 μl of saline. Additional ligand-uptake competition experiments were performed with cold choline (Sigma-Aldrich, St. Louis, MO, USA) to evaluate its influence in the cellular radiopharmaceutical uptake. An excess of cold choline was added to the cell culture medium 12 h before incubation of ^{18}F-FCH, to obtain a final concentration of 3 mg/ml, three fold higher than EMEM concentration one.

Cell kinetic studies and uptake evaluation. The cellular radiotracers uptake was determined with a 3×3'' NaI(Tl) pinhole 16×40 mm gamma counter (Raytest, Straubenhardt, Germany); all measurements were carried-out under the same counting geometry (along with a standard source to check the counter performance), data were corrected for background and decay. The total radioactivity was counted when the radiotracer was added to the medium in each flask (time 0).

After 20, 40, 60, 90 and 120 min, the medium was harvested, the cells were rapidly washed three times with 1 ml PBS and radiopharmaceutical uptake for each sample was assessed.

All experiments were carried-out in duplicate and repeated three times. The uptake measurements were expressed as percentage of the administered dose of tracers per 2×10^5 cells after correction for negative control uptake (flasks with complete medium incubated with radiopharmaceutical, without cells).

Cell viability assay. At the end of quantitative gamma spectrometry, adherent cells were harvested with 1% trypsin-EDTA solution and supernatants with adherent cells were counted with Burker's chamber. Trypan Blue dye assay was performed to assess cell viability as a standard protocol (8).

Statistical analysis. *In vitro* binding experiments were conducted in duplicate at least two times. Data (means) were compared using parametric or non-parametric tests as appropriate. Differences were regarded as statistically significant for $p<0.05$. Values are expressed as mean (with confidential interval, calculated with a significance level of 95%, CI 95%). Figures reported uptake of radiopharmaceutical as function of incubation period: values are shown as percentage of administered dose per 2×10^5 cells (mean±CI 95%). Therefore if error bars on the Y axis don't overlap each self, the two points are considered significantly different.

Results

Radiopharmaceuticals binding assay. In T98G glioma cells the ^{18}F-FCH was taken-up significantly after 60 min with a radioactivity percentage in the cells of 1.8±0.3%, 3.6±0.4%, and 3.6±0.6% at 60, 90, 120 min respectively. After 40 min incubation with 18F-FDG a peak of 4.0±1% of the administered dose was accumulated in the cells, but it didn't significantly differ from the ^{18}F-FCH 40 min uptake. Then the ^{18}F-FDG related uptake decreased by approximately three times the ^{18}F-FCH one. So, the comparison between two radiopharmaceuticals showed ^{18}F-FCH activity bound to T98G cells higher than ^{18}F-FDG one over 60 min incubation time (Figure 1).

Fibroblasts seemed not to accumulate neither ^{18}F-FDG nor ^{18}F-FCH specifically: at each incubation time the percentage of administered dose in the cells was lower than 1% (Figures 2 and 3).

Regarding ^{18}F-FCH, fibroblasts uptake was significantly different from the one for T98G cells throughout all incubation times (Figure 3). The difference in uptake was not evident with FDG (Figure 2). As a negative control, flasks containing medium without cells, incubated under similar conditions, showed a non-significant presence of radiotracers.

In binding competition experiments with the cold choline added to the medium of cell cultures, the uptake of ^{18}F-FCH was significantly lower, about 1% for all incubation times, similar to the uptake by fibroblasts (Figure 4).

Cell viability. Exposure to gas mixture was maintained throughout the experiment and cell viability was attested around 90%, under all experimental conditions (data not shown).

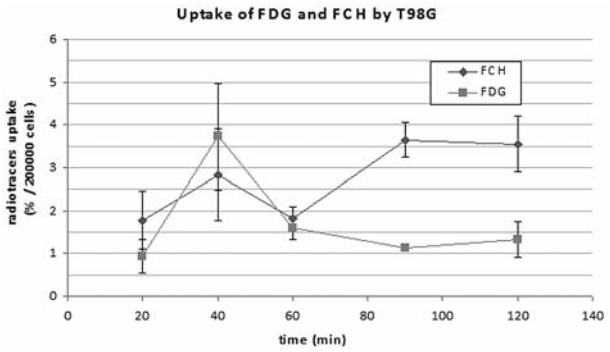

Figure 1. *Uptake of ^{18}F-FDG and ^{18}F-FCH by the T98G cell line.*

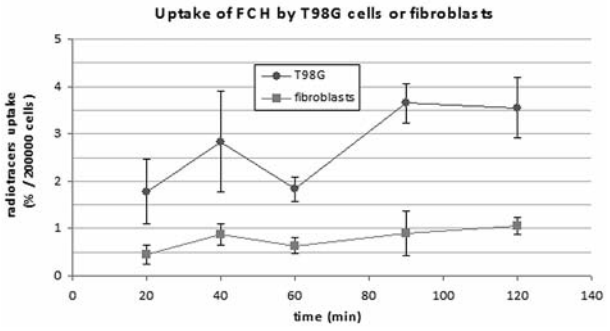

Figure 3. *Uptake of ^{18}F-FCH by T98G cells or fibroblasts.*

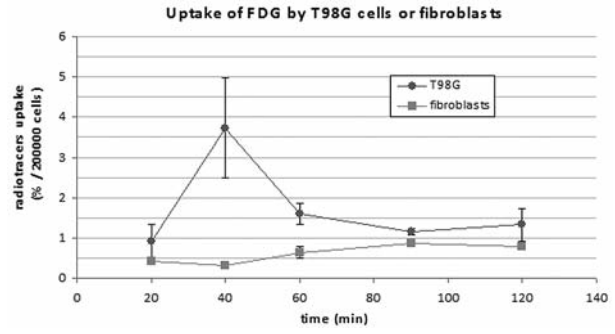

Figure 2. *Uptake of ^{18}F-FDG by T98G cells or fibroblasts.*

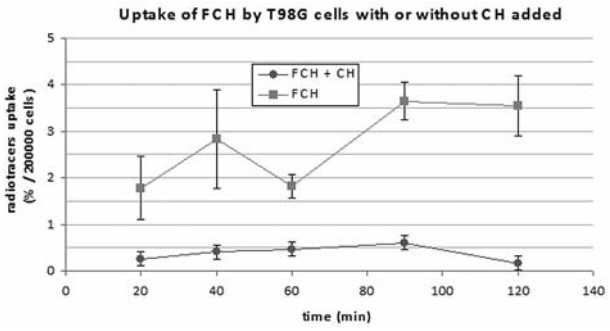

Figure 4. *Saturation binding test with cold choline in T98G cells binding sites.*

Discussion

Non-invasive PET imaging plays an important role in brain cancer evaluation. It is based on a radiotracer preferential accumulation in tumoral lesions compared to non-tumoral tissues, reaching the desired high tumor-to-background ratio. Distinguishing recurrent or residual disease from chemotherapy or radiation-induced injury remains a relevant clinical problem in choosing the optimal therapeutic regimen.

The higher up-regulated choline kinases activity typical of neoplasms, together with its practical supplying as "ready for clinical use" fluorinated compound, explain the role of fluorocholine as an oncological probe (9, 10), even though available data related to human brain tumors are still limited. In fact the CH analogue ^{18}F-FCH is considered an optimal PET radiotracer since on the basis of structural similarity it mimics CH uptake and metabolism well resembling the physiological processing of natural choline (6). Rottemburger found a high CH sensitivity and specificity in detecting brain metastases too (11). Its uptake correlation with cellular malignancy (12) and grade (3) clears-up the higher accumulation in chemo-radio-resistant cells (T98G) than in less aggressive ones, explaining our cell line choice. So, the T98G human glioblastoma cell line appeared to be a relevant model for interpreting the diagnostic power of *in vivo* scan. Our results show a significant affinity for glioma cells in comparison to non-neoplastic fibroblasts used as controls with both tracers (Figure 2), even though significant differences are observable related either to the uptake entity and the time activity course.

As reported in Figure 1 an apparent peak activity is observable in glioma cells at 40-min incubation either with FDG and FCH, however these data points are associated to a large standard deviation and this may likely be due to chance. After 1 h incubation FCH shows a clearly increased uptake in comparison to FDG, thereafter reaching a plateau, while the FDG uptake remains substantially unchanged or tends to a slow decrease up to 2 h. The FCH plateau activity may be in part spurious, because it has been reported in a pulse-chase experiment that a significant percentage of radioactivity can be cleared from cells demonstrating intracellular tracer de-phosphorylation (6). In comparison to

published results obtained with FCH in glioma cell lines, the uptake we observed is higher than reported by Bansal *et al*. (6): about 6%/10^6 cells in the less aggressive 9L cell line at 120 min and by Vanpouille *et al*. (5): between 0.8 and 1.3 %/2×10^5 U87MG cells and derived lines, even if in the latter-case comparison is more difficult due to the lack of information on the exact incubation time (0-90 min). So, the T98G human glioblastoma cell line appears to be a suitable model for interpreting the diagnostic power of *in vivo* scan and its behaviour further confirms clinical PET results suggesting a higher uptake in more aggressive tumors.

The fibroblasts FCH uptake is significantly lower in comparison to glioma cells, after 90 and 120 min incubation the ratio glioma/fibroblast is about three-fold. On the other hand, at the same incubation times FDG uptake in glioma cells is only slightly higher than in fibroblasts, the ratio being lower than 1.

From the competition experiments, after binding site saturation with cold choline, a lower cellular uptake of ^{18}F-FCH emerged. These findings further confirm that choline is transported by membrane saturable carriers, so this transport system is specific for the uptake of radiolabelled choline analogues in tumor cells (13), confirming ^{18}F-FCH as an optimal tracer for neoplastic lesions. In the comparison between fibroblasts and tumor cell uptake, a relevant difference was observed only with ^{18}F-FCH. Our choice of using fibroblasts as experimental healthy tissue model derives from the fact that they are an easily achievable essential component of ubiquitary connective tissues. They well-represent the blood vessels wall constitutive elements which are one of the three type of tissue in central nervous system affected by chemo-radio necrosis.

These results show that fibroblasts are unable to accumulate both ^{18}F-FCH and ^{18}F-FDG, but T98G glioma cells accumulate much more 18F-fluorocholine than ^{18}F-fluorodesoxyglucose. Thus, ^{18}F-FCH is confirmed as able to discriminate neoplastic cells from non-pathological cells, so "from bench to bed", better than ^{18}F-FDG in differentiating tumor lesions from surrounding tissue. Our results are in apparent disagreement with those of Boelcan *et al*. (14) in the F98 glioma rat model indicating a lower ^{18}F-FCH tumor to background ratio in comparison to ^{18}F-FDG. However, despite the limits of a comparison of *in vitro* results with those obtained in a animal model, uptake evaluation was performed early during the first 10 min after injection, while our results indicate a later accumulation of 18F-FCH in glioma cells achieving a more prominent ratio with normal fibroblasts after 90 min of incubation. It remains open to question whether ^{18}F-FCH can aid the diagnosis of radionecrosis. Boelcan *et al*. (14) did not find any significant differences between glioblastoma and radionecrosis, probably due to the high uptake by macrophages in his delayed radionecrosis animal model. This is in contrast with a previous study by Spaeth *et al*. (13) who found a significant difference in acute radionecrosis, probably characterized by less activated inflammatory response.

In conclusion, results provide evidence of the usefulness of ^{18}F-FCH as promising tracer, better than ^{18}F-FDG to underline the tumor to background ratio in brain tumors, although a direct translation of our *in vitro* data to human application need further investigation. Additional studies on radiation-injured cells and radiation necrosis-associated cells will be carried-out in order to test the potential role of FCH in the differential diagnosis.

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