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Full Paper

L-Amino acid load to enhance PET differentiation between tumor and inflammation: an *in vitro* study on ^{18}F-FET uptake

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ABSTRACT: Labeled amino acids (AA) are tumor tracers for use in nuclear medecine. *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine (FET) is transported by the L-system, known to function as an exchanger. *In vitro* utilization of FET, after a preload or prior to an afterload of non radioactive L-amino acids, was evaluated in order to measure the potential effects of AA content on the distinction between tumor and inflammatory lesions. Cellular uptake of FET was studied on rat osteosarcoma cells (ROS 17/2.8) and human leukocytes, initially loaded with nonradioactive L-tyrosine or L-methionine. FET efflux was evaluated from cells loaded with nonradioactive L-phenylalanine after tracer uptake. ROS 17/2.8 showed a higher sensitivity to preload and afterload effects on cellular FET content as compared with the leukocytes. We conclude that preload with L-tyrosine, prior to the administration of FET, may be a potential procedure to improve PET differentiation between tumor and inflammatory lesions. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: amino acid; preload; afterload; tumor; inflammation; PET; FET

INTRODUCTION

Diagnosis and treatment in oncology are based on precise tumor detection, which requires differentiation between normal and neoplasic tissues, as well as between tumor and inflammatory changes.

Tumor cells are characterized by enhanced glycolysis and glucose transport (1–7). These biological features have led to the extensive application of PET in oncology, by using 2-[^{18}F] fluoro-2-deoxy-D-glucose (FDG), a sensitive tracer of a very large variety of cancers (8). The major drawback of FDG in oncology is related to the high affinity of inflammatory cells for this tracer (9–11). This feature reduces its diagnostic specificity, particularly in situations in which a differential diagnosis need to be made between malignant tumors and benign inflammatory lesions. This is typically the case for lesions localized in the lungs or the head–neck area, in which infectious lesions and inflammatory lymph node reactions are frequent. In addition, FDG uptake in inflammatory cells may also complicate the interpretation and quantification of the FDG signal within a tumor area since tumors are frequently infiltrated by various amounts of inflammatory cells (11,12). Efforts have therefore been put in the development of tracers that better differentiate tumor from inflammation. As amino acids (AA) are highly metabolized and transported into tumor cells (13,14), several tracers based on their structures have been proposed. For some of them, the uptake in inflammatory cells has been reported as low, compared with FDG (15,16), even if this advantage has been challenged in case of brain lesions (17,18). Labeled AA PET tracers developed so far can be divided into two categories. Some tracers are actively incorporated into the proteins, like [^{11}C]methyl-L-methionine (MET) (13,19–22), 2-[^{18}F]fluoro-L-tyrosine (FT) (13,21,23–25) and 4-[^{18}F]fluoro-L-phenylalanine (FPhe) (13), potentially allowing studies of protein synthesis rate, while others, like 2-[^{18}F]fluoro-α-methyl-L-tyrosine (FMT) (13,26) and *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine (FET) (13,27–33), are not integrated into proteins and are therefore valuable tools to specifically evaluate the AA transport process. FET uptake is reported to be particularly elevated in squamous cell carcinomas, a feature of interest since those carcinomas are frequent in lung and head–neck localizations in which differentiation from inflammatory diseases is critical (31).

AA translocation inside the mammalian cell is mediated through transporter systems, which are divided in two major categories: sodium-dependent and sodium-independent transporters (34,35). They have overlapping

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specificities. Among the sodium-dependent transporters, system *A* is specific for AA with short polar or linear side chains (alanine, glycine and methionine) (36). Among sodium-independent transporters, the L-system is associated with branched, aromatic or neutral AA (leucine, isoleucine, valine, phenylalanine, tyrosine and methionine) (34,37–39).

The L-system is an obligatory exchanger; it brings a sterically hindered AA inside the cell through an exchange with an intracellular AA. As suggested in previous studies (38,40–42), this property can be exploited to improve the uptake of extracellular radiotracers thanks to a cellular preload with AA. Indeed, exposing cells to increased extracellular concentrations of AA leads to increased intracellular AA concentrations, and the large amount of AA accumulated in the cell is then available for further trans-membrane exchange. AA preload therefore accelerates the exchange mechanism that leads to the intracellular uptake of radioactive tracers transported by the L-system (43,44). The uptake kinetics of labeled tracers markedly depend on their structure so that the effect of cellular preload on their uptake, within the time frame of a human PET study, may vary between these tracers. Another procedure making use of the exchange mechanism of the L-system is the afterload of AA following the tracer administration (42). Indeed, exposing the cells to high concentrations of exchangeable AA after tracer incorporation will facilitate tracer clearance from the free intracellular pool, wherever tracer transport depends on L-system activity. Comparing cellular radioactivity before and after post-incubation exposure to nonradioactive AA will therefore test the L-system implication in the tracer uptake mechanism.

The transport systems involved in AA-related tracer uptake certainly vary as a function of the cell type since they depend on the differential expression or over-expresssion in the case of cancer cells of various transporter units and sub-units (22,25,45). On the other hand, AA transport systems not based on an exchange mechanism are insensitive to the so-called trans-stimulation effects produced by AA preload and afterload. The present study aimed at assessing if differences in kinetics between AA tracers uptake in tumor and inflammatory cells may be enhanced by trans-stimulation of the transport. The practical goal targeted is the definition of imaging protocols that may help in the differentiation between malignant and inflammatory lesions.

MATERIALS AND METHODS

Products and materials

Acetonitrile, diethylene glycol ditosylate, L-methionine, DMSO, cation exchanging cartridges 1 ml/50 mg and a semi-preparative Discovery C_{18} column 5 μm, 250 × 10 mm were purchased from Sigma-Aldrich (Bornem, Belgium). $[^{18}O]H_2O$ was purchased from Rotem (Beer Sheva, Israel), Kryptofix 2.2.2., K_2CO_3 and TLC silica plates from Merck (Leuven, Belgium), phosphate buffered saline (PBS), modified Eagle's medium (MEM) and foetal calf serum (FCS) from Cambrex (Verviers, Belgium), L-tyrosine, NaOH pellets, Na_2CO_3, dichloromethane and diethytlether from Acros Organic (Geel, Belgium), L-phenylalanine from Activotec (Cambridge, UK), silica Sep Pak cartridges and anion exchanging cartridges from Waters (Zellik, Belgium), Millipore GS 0.22 μm filter from Millipore (Brussels, Belgium) and Ficoll-Paque from Amersham Biosciences, (Uppsala, Sweden). HPLC analyses were performed using a Waters Alliance 2695 system (Zellik, Belgium), equipped with a Beckman Ultrasphere C_{18} column, 250 × 4.6 mm (Weilheim, Germany). TLC plates were analysed using a Tracemaster 20 automatic TLC linear analyser from Berthold (Vilvoorde, Belgium) and the osmolarity was measured using an automatic osmometer, the micro-osmometer 13/13 DR, from Roebling (Berlin, Germany). Cellular radioactivity was counted in an auto gamma-counter Cobra II from Perkin Elmer (Brussels, Belgium).

Synthesis of FET

Fluorine-18 in the form of fluoride was prepared by bombardment of $[^{18}O]H_2O$ (2 ml, 99% enrichment), with 15 MeV protons using a Cyclone-30 cyclotron (Ion Beam Applications, Louvain-la-Neuve, Belgium). After bombardment, the target liquid was led over an anion exchange column and the ^{18}F-fluoride was eluted in a 24 ml reaction vial with an aqueous solution (1 ml) of $[K_2CO_3/K_{2.2.2.}]$. The eluate was dried by addition of acetonitrile, under a stream of argon. A solution of 1,2-ditosyloxyethane (5 mg) in CH_3CN (1 ml) was mixed with $[K_2CO_3/K_{2.2.2.}]/[^{18}F]F^-$ complex and the mixture was heated for 10 min at 70°C. The reaction mixture was then applied on a silica SepPak cartridge and, after elution of the solvent and drying of the column with a stream of nitrogen, [^{18}F]fluoro-ethyltosylate ([^{18}F]FEtOTs) was eluted with 10 ml of anhydrous diethylether. The [^{18}F]FEtOTs was mixed with unprotected L-tyrosine (2 mg) for 15 min at 90°C in DMSO (0.5 ml)–NaOH. The reaction mixture was directly injected onto a semi-preparative C_{18} column (water–ethanol–acetic acid 87.5:10:2.5 containing 3.2×10^{-2} M of ammonium acetate at a flow rate of 4 ml/min). The fractions containing FET were collected at 6.4 min and were loaded on a strong cation exchanging cartridge and eluted with PBS (5 ml). The pH was adjusted to 7 with 0.5 M solution of Na_2CO_3 (1 ml). After a sterile filtration on

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Millipore GS 0.22 μm filter, the final product was identified by reversed-phase HPLC.

Cell culture

Stock cultures of rat osteosarcoma cells (ROS 17/2.8) were maintained in MEM, supplemented with 10% FCS. The cells were harvested by trypsinization and washed three times with PBS. Human mononuclear blood cells were prepared from a 20 ml volume of heparinized blood. The blood was mixed with an equal volume of 0.9% NaCl and layered onto Ficoll-Paque in 50 ml conical tubes (20 ml of diluted blood on 12 ml Ficoll-Paque). The tubes were centrifuged at 400 g for 25 min at room temperature. The mononuclear cells were harvested from the interphase using a Pasteur pipette, resuspended with 10 ml PBS and centrifuged at 200 g for 12 min. The cell pellet was then washed three times with 10 ml PBS (150 g, 5 min). Cells were counted with a hemocytometer. Viability was assessed with the trypan blue exclusion method. A total of 2×10^6 cells were used in each experimental tube.

Preload protocol

ROS 17/2.8 and human leukocytes were preloaded by incubation for 15 min at 37°C with different concentrations of AA (L-tyrosine or L-methionine). Cells were then centrifuged (5 min at 150 g) and washed. The pellet was suspended in PBS. FET (=14.8–18.5 MBq) was added and the incubation was prolonged for 30 min at 37°C. Cells were finally washed three times with PBS. Pellets and supernatants were counted in an auto gamma-counter Cobra II in order to estimate the tracer uptake expressed as percentages of the activity.

Afterload protocol

Cells were washed three times with cold PBS after the preload protocol and the FET uptake process described above, in order to reduce interference from remaining L-tyrosine or FET in the extracellular medium. The cellular pellet was suspended in 2 ml of PBS or 0.25 mM L-phenylalanine during 30 s, 1 min or 4 min. The incubation was stopped by adding cold PBS solution. The preparation was centrifuged at 150 g at 4°C, and then the pellet was separated from the medium and suspended in PBS. The tracer efflux was estimated from the activity counted in the pellet and in the medium and expressed as a percentage of the incorporated activity.

Statistical methods

Chemical and radiochemical yields were expressed as mean ± SEM. The comparisons were tested by using Student paired and nonpaired *t*-test.

RESULTS

Synthesis of FET

The chemical yield of 1,2-ditosyloxyethane [^{18}F]fluorination was 61 ± 2% and the radiochemical purity was 75 ± 2%. The [^{18}F]fluorethylation of unprotected L-tyrosine was achieved with a chemical yield of 66 ± 4%. The final product was obtained with a radiochemical yield (end-of-beam, decay corrected) of 13 ± 1% ($n = 17$), a radiochemical purity of 92 ± 1% and a specific activity of 21 ± 1 GBq/μmol.

FET uptake in ROS 17/2.8 and human leukocytes after a L-tyrosine preload

In ROS 17/2.8, FET uptake was clearly modulated by a preload of the cells with L-tyrosine (Fig. 1): it increased progressively with increasing L-tyrosine concentrations from 0.25 to 1 mM; it returned to basal values at 2 mM L-tyrosine; and it was markedly decreased at 2.7 mM. In leukocytes, no effect on FET uptake was observed at concentrations ranging from 0.25 to 1 mM. After a preload with 2.7 mM of L-tyrosine, the uptake was markedly decreased, as observed with ROS 17/2.8. The uptake of FET was higher in tumor cells compared with leukocytes in all experimental conditions (from 0 to 2 mM of preloaded L-tyrosine), but the maximum difference between tumor cells and leukocytes occurred when cells were preloaded with 1 mM of L-tyrosine (Fig. 2).

FET efflux from ROS 17/2.8 and leukocytes after an afterload of 0.25 mM of L-phenylalanine

As illustrated in Figs 3 and 4, only ROS 17/2.8 cells were responsive to an afterload with 0.25 mM of L-phenylalanine, which slightly increased the efflux of FET from ROS 17/2.8 in all experimental conditions (with or without preload with L-tyrosine, variable duration of efflux step). No significant effect of an afterload with L-phenylalanine was observed on the FET efflux from leukocytes. Differences between the effects on ROS 17/2.8 and leukocytes have been evaluated in two separate experiments by FET content ratio in both cell types (Table 1). The cells were initially preloaded or not with L-tyrosine (1 mM) before tracer administration. The activity ratio between ROS 17/2.8 and leukocytes was decreased when an afterload of L-phenylalanine was applied (paired *t*-test: $p = 0.01$).

FET influx in ROS 17/2.8 and leukocytes after a L-methionine preload

No significant effect of a preload with increasing concentrations of L-methionine (0.25–2.5 mM) was

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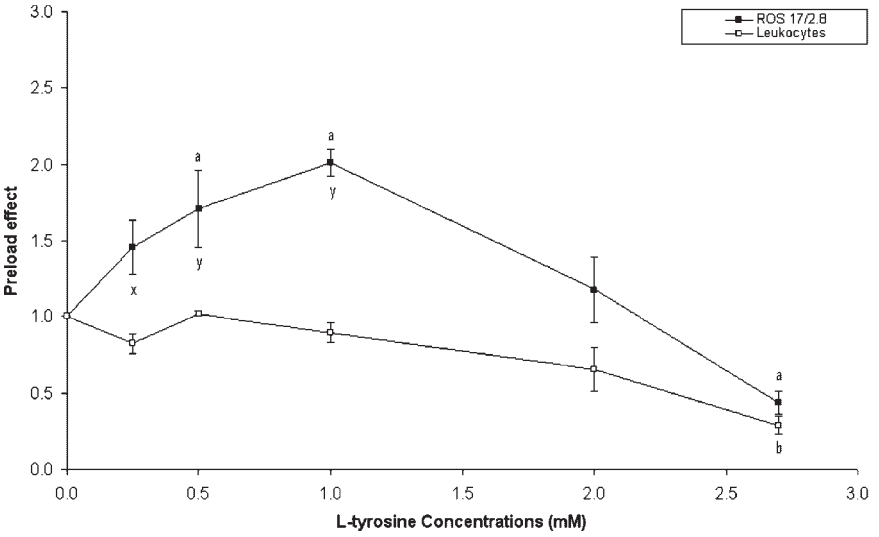

Figure 1. Preload effect on FET uptake in ROS 17/2.8 cells and leukocytes as a function of L-tyrosine concentrations. Results (mean of three separate experiments ± SEM) are expressed as the ratio between the tracer uptake into preloaded cells and control cells (0 mM of L-tyrosine). L-Tyrosine effect on FET uptake by ROS 17/2.8 is significant at different concentrations (a, nonpaired Student's *t*-test: $p < 0.01$). L-Tyrosine effect on FET uptake by leukocytes is significant at the highest concentration (b, $p < 0.01$). Student test between ROS 17/2.8 and leukocytes at each L-tyrosine concentration showed that significant differences between ROS 17/2.8 and leukocytes occured at low concentrations of L-tyrosine (*x*, $p < 0.05$; *y*, $p < 0.001$).

observed, neither in ROS 17/2.8 nor in leukocytes (Fig. 5). When the washing steps between the preload and tracer administration were suppressed, the FET uptake was strongly inhibited, even with the lowest concentration of L-methionine (data not shown).

DISCUSSION

Synthesis of FET

For the radiosynthesis of FET, we slightly modified the original method described by Wester *et al.* (14), a two-

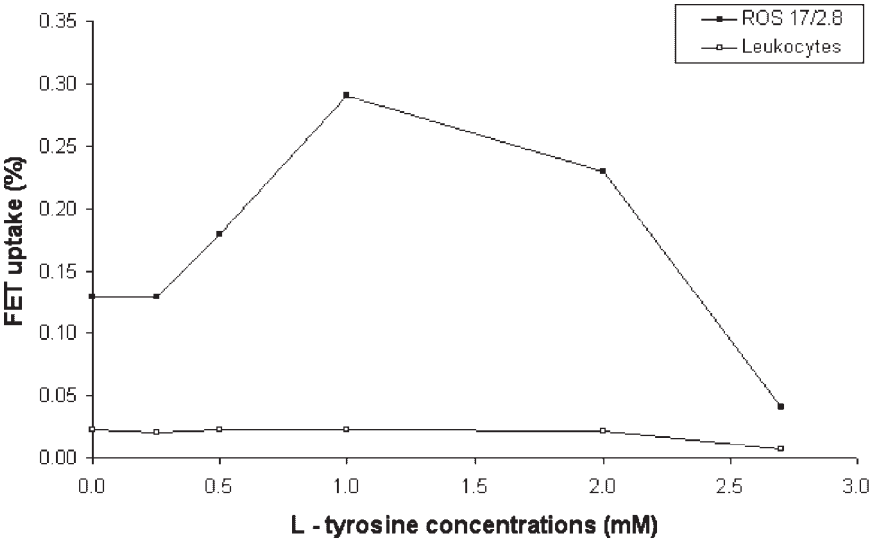

Figure 2. FET activity recovered from ROS 17/2.8 and leukocytes (expressed as a percentage of loaded activity) as a function of L-tyrosine concentrations used for the preload in a representative experiment. The difference between ROS 17/2.8 and leukocytes was significant (paired Student's *t*-test: $p = 0.007$ in this experiment).

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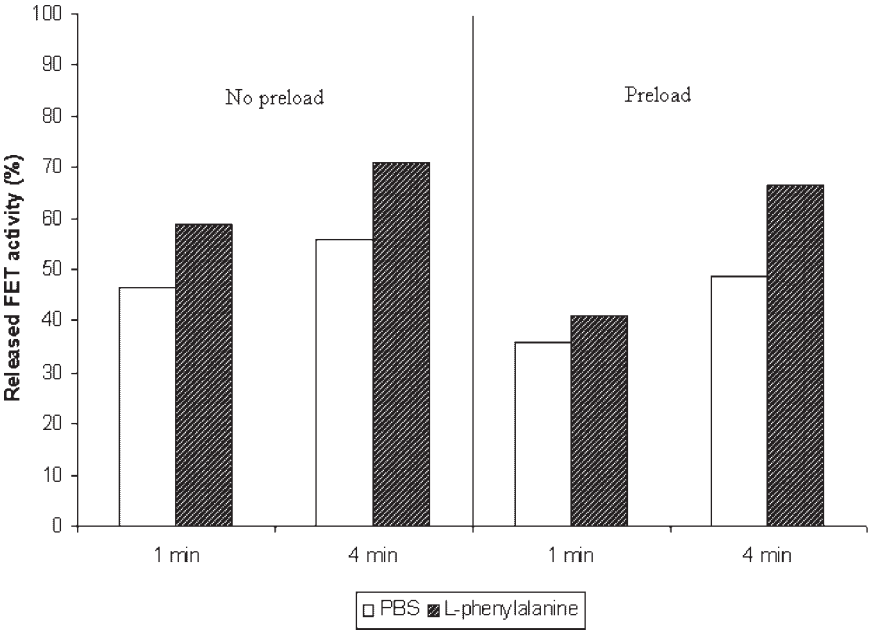

Figure 3. FET release (as a percentage of incorporated activity) by ROS 17/2.8 in the presence or absence of L-phenylalanine in a representative experiment. The cells were initially preloaded or not with L-tyrosine (1 mM) before the tracer administration. Results are presented for two afterload incubation times (1 and 4 min). The paired Student's *t*-test shows a significant effect of L-phenylalanine ($p < 0.05$ in this experiment, and $p < 0.001$ for all grouped experiments).

step reaction sequence, consisting of the [^{18}F]fluorination of 1,2-ditosyloxyethane and the subsequent [^{18}F]fluorethylation of protected L-tyrosine. At variance with the original method, we used unprotected L-tyrosine and did not proceed to an intermediate HPLC purification of the labeling agent [^{18}F]FEtOTs. This simplified procedure led to a chemical yield that was comparable to the one obtained with the original method (66% instead of 75%).

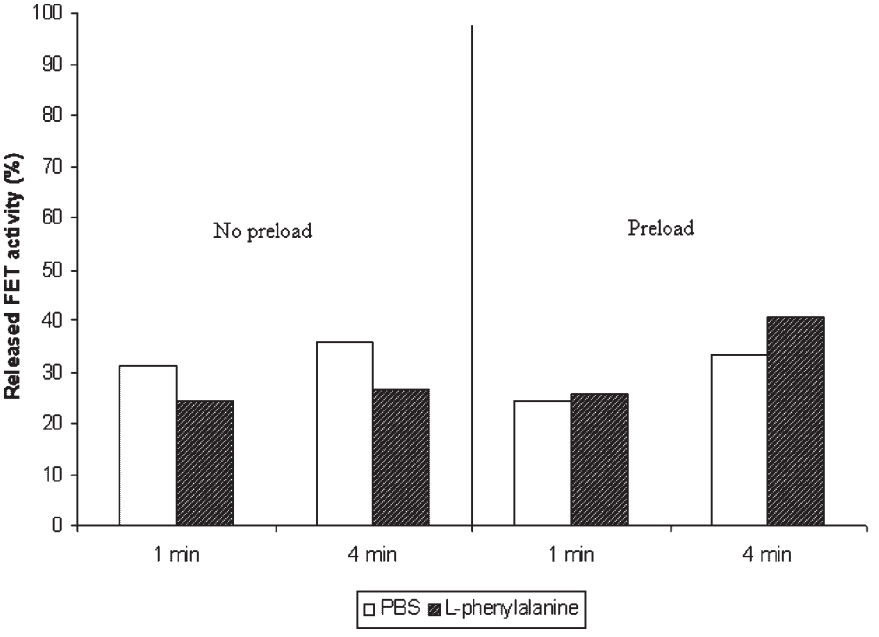

Figure 4. FET release (as a percentage of incorporated activity) by leukocytes in the presence or absence of L-phenylalanine in a representative experiment. The cells were initially preloaded or not with L-tyrosine (1 mM) before the tracer administration. Results are presented for two afterload incubation times (1 and 4 min). There was no significant effect of L-phenylalanine in this experiment and for all grouped experiments.

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Table 1. Ratio of FET content in ROS 17/2.8 and leukocytes after an afterload with L-phenylalanine or PBS[a]

		Experiment 1		Experiment 2	
	Afterload duration (min)	Afterload PBS	Afterload PHE	Afterload PBS	Afterload PHE
Preload PBS	0.5	10.1	7.5	ND	ND
Preload TYR	0.5	29.9	14.8	ND	ND
Preload PBS	1	ND	ND	3.4	2.3
Preload TYR	1	21.2	14.1	7.1	5.4
Preload PBS	2	9.7	6.1	ND	ND
Preload TYR	2	19.2	13	ND	ND
Preload PBS	4	ND	ND	3	1
Preload TYR	4	11.9	9.5	5.5	3.8

PBS, phosphate-buffered saline solution; PHE, L-phenylalanine; TYR, L-tyrosine; ND, not determined.
[a]Ratio between ROS 17/2.8 and leukocytes is decreased when an afterload of L-phenylalanine is applied (paired Student's *t*-test, $p < 0.01$).

FET influx in ROS and leukocytes after a L-tyrosine preload

Large neutral AA are mainly transported inside the cell by the L-transporter, which functions as an obligatory exchanger system (34,35). In view of this particular property, preloading cells with exchangeable AA has been suggested for AA PET imaging in order to boost L-system activity, and so to increase the uptake of extracellular labeled tracers (38,40–42). When we preloaded the osteosarcoma cells with different concentrations of L-tyrosine, we observed a progressive increase of FET uptake until a concentration of 1 mM of L-tyrosine is reached. At higher concentrations of L-tyrosine, the uptake of FET returned to basal levels and it was finally inhibited above 2 mM. The cause of this inhibition is probably to be found in the experimental conditions. The washing steps following the preload process removes most of the L-tyrosine present during the preload. However, at the highest concentrations, the L-tyrosine remaining in the extracellular environment appeared to be sufficient to compete with the influx of FET. Also, L-tyrosine massively accumulated in the cells during the preload is released by exchange with FET and may compete secondarily with remaining extracellular FET. This competition by released L-tyrosine is facilitated by the slow kinetics of the FET uptake process, as compared with the uptake of natural AA.

In our experimental conditions, FET uptake in leukocytes was not modulated by a preload of the cells with L-tyrosine. The difference in AA transporter expression by tumor and inflammatory cells probably explains the differences observed in AA preload effects.

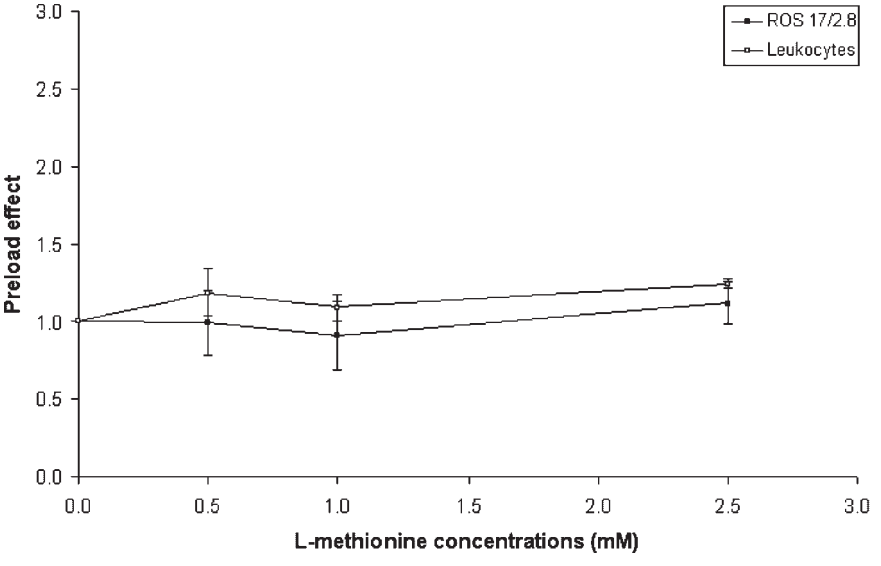

Figure 5. Preload effect on FET uptake in ROS 17/2.8 and leukocytes as a function of L-methionine concentrations. Results (means of three separate experiments ± SEM) are expressed as the ratio between the tracer uptake into preloaded cells and control cells (0 mM of L-methionine). There was no significant effect of L-methionine preload.

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Because the subtype hLAT2 of the L-system has been essentially identified in tumor cells (45), and not in inflammatory cells, this sub-type of transporter may play an important role in FET uptake in tumor cells.

FET efflux from ROS 17/2.8 and leukocytes after an afterload of 0.25 mM of L–phenylalanine

Taking advantage of the exchange properties of the L-transporter and its high expression on tumor cells, we examined if a transportable substrate of the L-system (i.e. L-phenylalanine) could differentially affect the efflux of loaded FET in osteosarcoma cells and leukocytes. We expected a massive efflux of FET from ROS 17/2.8, and a minor effect on leukocytes, thus providing a potential method of differentiation between malignant and inflammatory lesions in clinical PET. In our experiments, leukocytes were not sensitive to the L-phenylalanine afterload while, in the osteosarcoma cells, FET efflux was actually increased in this experimental condition, although the response of malignant cells to L-phenylalanine afterload remained insufficient to envisage valuable translation in clinical settings. The reasons for such a limited effect of the L-phenylalanine afterload on tumor FET content remain uncertain. The kinetics of tracer wash-out in the baseline conditions has probably precluded the demonstration of larger effects of the AA afterload.

FET influx in ROS 17/2.8 and leukocytes after a L-methionine preload

Since the efficiency of a preload with L-tyrosine appeared to be limited by a competitive effect at high concentrations of L-tyrosine, we tested another AA, L-methionine, which has an affinity for both *A* and L-systems. The transport of L-methionine has been described as 'asymmetric' (38,42,46,47): L-methionine enters exclusively by the unidirectional *A* system and leaves the cell by the L-system. This property should allow exchange of L-methionine with FET, without competition, altering FET entry through the L-system. We observed no effect of L-methionine preload, indicating that regulation of the slow transport of non-natural AA such as FET was not fully mastered by the theoritical model proposed and the experimental settings that we have adopted.

Study limitations and clinical perspectives

The present *in vitro* study has some limitations that preclude firm conclusions on potential translation in clinical practice. Indeed, inflammation involves different types of immune cells and the balance between the different cell types may vary during the course of inflammatory conditions and as a function of their pathogenesis. Further studies should be conducted on specific cell types, such as the activated macrophages that are particularly enriched in granulomatous disorders. Indeed, AA transporter profile in inflammatory cells is expected to vary under the influence of various cytokines present in inflammatory and malignant lesions (30). Also, expression profile of AA transporters may vary in different cancers so that effects revealed in a sarcoma cell line cannot be directly extrapolated to cancers of a different origin. Also, in the clinics, variations in nutritional status and AA serum profile may affect the impact of procedures that bases the differentiation between tumor and inflammatory lesions on transstimulation effects. The actual blood level of AA that may be reached in AA challenge applicable in the clinics is about four times lower than the extracellular concentrations used in the present *in vitro* experiments but the kinetics of the exposure would be very different, precluding direct *in vitro* to *in vivo* extrapolation. Still, an AA exposure similar to what is applicable in humans has shown transstimulation effects in a rat model of sarcoma (42). AA preload procedures in patients would certainly affect our capacity to quantify tracer uptake in absolute terms. This is not a major issue since PET data analysis on AA uptake is usually limited to semi-quantification because full quantification models are difficult to validate in humans.

CONCLUSIONS

We have demonstrated that an L-tyrosine pre-incubation load modulates FET uptake in cancer cells. Such an effect was not observed in inflammatory cells. An increased differentiation between tumor and inflammation can therefore be expected in clinical PET protocols that include an AA preload procedure. In our *in vitro* experiments, we determined an optimal preload concentration of 1 mML-tyrosine. The L-methionine preload was unable to improve FET uptake in tumor cells. The L-phenylalanine afterload attenuated FET uptake in the tumor but not the inflammatory model. Complex clinical protocols involving dual PET acquisitions could take profit of this differential behavior between tumor and inflammatory cells. Still, the magnitude of the differentiation obtained in our *in vitro* experiments remains too weak to encourage AA afterload for the tumor vs inflammation differentiation.

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