Uptake of [18F]fluorodeoxyglucose in human monocyte-macrophages in vitro

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Abstract. The fact that fluorine-18 fluorodeoxyglucose ([18F]FDG) accumulates in inflammatory lesions as well as in tumours reduces the diagnostic specificity of positron emission tomography (PET) in oncology. The aim of this study was to characterise the uptake of [18F]FDG in isolated human monocyte-macrophages (HMMs) in vitro in comparison with that in human glioblastoma (GLI) and pancreatic carcinoma cells (PAN). The purity of HMM preparations was determined by immunohistochemical staining and their functional integrity was assessed by long-term incubation with iodine-131 acetylated bovine serum albumin. [18F]FDG uptake in HMMs was quantified as percent of whole [18F]FDG activity per well (% ID) or as % ID in relation to total protein mass. [18F]FDG uptake in HMMs significantly increased with culture duration, yielding 7.5%±0.9% (% ID/100 µg) at day 14. Stimulation by lipopolysaccharide further enhanced [18F]FDG uptake in HMMs by a factor of 2. [18F]FDG uptake significantly decreased with increasing glucose concentration in the medium. Radio-thin layer chromatography of intracellular metabolites revealed that [18F]FDG was trapped by HMMs mainly as [18F]FDG-6-phosphate and [18F]FDG-1,6-diphosphate. [18F]FDG uptake was in the range of uptake values measured in GLI and PAN. By accumulating [18F]FDG in a manner analogous to uptake by tumour cells, activated HMMs may contribute to the [18F]FDG uptake values measured by PET in neoplasms.

Keywords: FDG – Macrophages – Inflammatory cells – Lipopolysaccharide – PET

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Introduction

Fluorine-18 fluorodeoxyglucose positron emission tomography ([18F]FDG-PET) is being successfully utilised as a cost-effective modality for the management of patients with various malignancies [1, 2]. However, high [18F]FDG uptake is not specific to cancer, but is also found in inflammatory lesions [3, 4, 5]. This may reduce the diagnostic specificity of [18F]FDG-PET in oncology; it is, however, also the basis for its use in the localisation of inflammatory foci [6, 7].

It is assumed that [18F]FDG uptake in inflammatory tissue is due to accumulation in inflammatory cells such as macrophages [3], lymphocytes [8] and granulocytes [9]. Among these, macrophages derived from blood monocytes play an important role as they are involved in the clearance of unphysiological proteins, cellular debris and apoptotic cells. Moreover, [18F]FDG uptake in inflammation has been studied in different animal models [5, 8]. In these models, macrophages and lymphocytes are often held responsible for high [18F]FDG accumulation. In abscesses, for example, they are regularly found in the so-called marginal zone [5].

Recently, in vitro models of neutrophil granulocytes and lymphocytes have led to a closer knowledge of [18F]FDG uptake mechanisms in these inflammatory cell types under different activation conditions [8, 9]. However, [18F]FDG uptake in isolated macrophages has not been systematically investigated. Therefore, the aim of this study was to characterise the uptake of [18F]FDG in a well-defined model of human monocyte-macrophages (HMMs) in vitro.

Materials and methods

Human tumour cells. Human primary pancreatic adenocarcinoma cells (BxPC-3, European Collection of Cell Cultures No: 93120816) and human glioblastoma cells (U-138 MG) were handled as described in the literature [10]. Pancreatic adenocarcinoma cells and glioblastoma cells were plated in 50-ml plastic culture

bottles. Approximately 200,000 cells were replated in 60-mm culture dishes 48 h before further experimental use.

Preparation and culture of human monocyte-macrophages. Human mononuclear cells were isolated from healthy human subjects by density gradient centrifugation using Ficoll methods [11] and macrophages were derived during cell culture as previously described [12]. Cells were resuspended in minimum essential medium (MEM, Gibco) containing penicillin (100 IU/ml) and streptomycin (100 IU/ml). Approximately 10^6 cells/ml were plated in 60-mm plastic culture dishes to generate a cellular monolayer with an optimally sized cellular surface for [^{18}F]FDG uptake. Cells were cultured in a humidified incubator in an atmosphere of 5% CO_2, 95% air (v/v) at 37°C. The monocytes were purified by allowing them to adhere to the plastic surface. Two hours after plating, the medium of the dishes and non-adherent cells, i.e. lymphocytes, were aspirated and adherent mononuclear cells were washed twice with MEM. MEM was replaced by macrophage culture medium (MSFM, Gibco) containing human serum [25% (v/v)] and penicillin/streptomycin (100 IU/ml). Cells were cultured in this medium for 4 days. Thereafter the medium was replaced by fresh MSFM containing human serum (10% (v/v) and penicillin/streptomycin (100 IU/ml). This medium was changed every 2 or 3 days. Unless otherwise indicated, cells were cultured for 12–15 days after plating before the experiments were initiated. Before the assays, HMMs were washed twice in PBS. Then HMMs were incubated for 1 h at 37°C in MEM. This incubation was performed to allow internalisation or dissociation of membrane-associated proteins or cell secretion products. Thereafter the medium was aspirated, cells were washed (PBS 1×), and the assays were initiated.

Cell viability and purity of human monocytes-macrophages. Cell viability was determined by trypan blue staining. Viability assays were performed in duplicate in cultures from five individuals. Cell viability exceeded 95% in all cases. In order to verify the purity of HMMs, a commercially available primary antibody against human granulocytes (MAK-BW-250/183, Immunomedics) was used in one representative HMM preparation. MAK-BW-250/183 was labelled with technetium-99m according to the instructions of the manufacturer. HMMs were incubated with ^{99m}Tc-MAK-BW-250/183 as described for [^{18}F]FDG uptake experiments.

Histochemical staining of human monocytes-macrophages. In five experiments, cells were classified as monocytes-macrophages by immunohistochemical staining using two primary antibodies [anti-CD14, anti-CD71 (Dako)] following the instructions of the manufacturer (APAAP-kit, Dako). More than 98% of peripheral mononuclear blood cells were classified as monocyte-macrophages.

Radiosynthesis of [^{131}I]Ac-BSA and functional analysis of HMMs. Acetylation and radioiodination of bovine serum albumin (BSA, Sigma) were done basically as previously described [13]. In a typical synthesis 2 mg BSA was acetylated by the procedure of Basu et al. [13]. The subsequent radioiodination was performed by adding 30 µCi (2–5 µl) n.c.a. Na^{131}I solution (IBSSO, Amersham) to a solution of 100 µg Ac-BSA in 120 µl phosphate buffer (pH 8). Radioiodination was started by adding 15 µl chloroamine-T (1 mg/ml). After 2 min reaction time, quenching was performed by the addition of 100 µl 50 m*M* sodium bisulphite in 2 *N* NaOH. [^{131}I]Ac-BSA can easily be separated by solid phase extraction using Bio-Gel P2 (Biorad) and subsequent ultracentrifugation (Micron-3, Amicon). The specific activity of [^{131}I]Ac-BSA was 700 Bq/µg. For functional analysis of monocytes differentiating to macrophages, the culture medium was supplemented with [^{131}I]Ac-BSA for 4 h at 37°C. The cellular uptake and degradation of [^{131}I]Ac-BSA were determined by precipitation with 10% trichloroacetic acid and radioactivity was measured in a gamma counter.

[^{18}F]FDG uptake experiments. [^{18}F]FDG was diluted in glucose-free medium. Four MBq [^{18}F]FDG was added to each culture well containing 1.2 ml MEM and incubation continued at 37°C for 3 h unless otherwise indicated. After incubation the medium was removed and an aliquot was used for radioactivity measurements. Subsequently, the cells were washed twice with PBS. Cells were dissolved from the wells mechanically and the resulting solution was transferred to tubes with a PBS rinse. After homogenisation, an aliquot was taken for radioactivity measurement (Caprac Counter, Capintec) and for protein determination by the method of Bradford [14]. [^{18}F]FDG activity was quantified as percent of whole [^{18}F]FDG activity (% ID) or as % ID divided by total protein mass. Cellular washout of [^{18}F]FDG and metabolites was determined at time intervals between 1 and 3 h by measuring the radioactivity concentration in the medium after washing with PBS and replacement by FDG-free MEM (1.5 ml/well).

Further incubation studies were directed at investigating the following parameters:

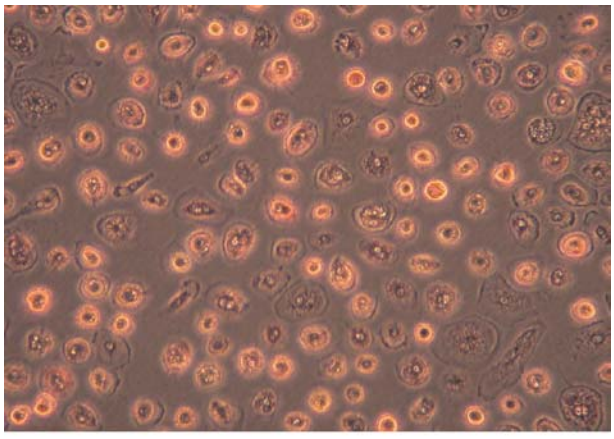
- Incubation time, varied between 15 min and 3 h
- Glucose concentration of MEM, varied between 0 and 1,000 mg/dl
- The effect of lipopolysaccharide (LPS), by pre-incubation with 8 µg/ml LPS for 24 h
- Culture duration, varied between 1 day and 14 days after the initiation of culture

Radio-thin layer chromatography. [^{18}F]FDG metabolites in HMMs were investigated by radio-thin layer chromatography on cellulose-coated plates (CEL 300, Macherey-Nagel) and ethanol/1 *M* NH_4OAc 3:2 (v/v) as previously described [15]. Chromatograms were measured using the Instant Imager (Canberra Packard), and metabolites were identified by their R_f values.

Statistics. All experiments were performed in triplicate unless otherwise indicated. Data are expressed as mean±SEM. The significance of differences in means was examined using Student's *t* test for paired data. To correlate two variables, Pearson's correlation coefficients were used. *P* values <0.05 were considered significant.

Results

More than 98% of isolated and purified cells expressed surface antigens CD14 and CD71 specific for monocytes-macrophages (Fig. 1). Moreover, the functional differentiation of monocytes to macrophages was characterised by the ability of the cells to endocytose unphysiological proteins through the involvement of scavenger receptors. In our study the ^{131}I-labelled unphysiological protein [^{131}I]Ac-BSA was used to depict increasing endocytosis during cell culture (Fig. 2). In addition, purity of the HMM preparation was confirmed by incubation with a ^{99m}Tc-labelled primary antibody against human granulocytes (MAK-BW-250/183). ^{99m}Tc activity specific for granulocytes measured in the HMM cultures was



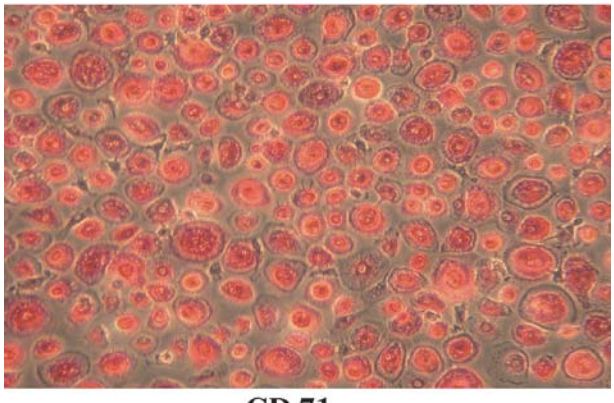


Fig. 1. Immunhistochemical classification of HMMs. Monocytes were isolated, purified and stained as described in Materials and methods. Cells were maintained in non-coated plastic dishes and underwent a process of differentiation to macrophages during cell culture. More than 98% of cells expressed the cell surface antigens CD14 (*top*) and CD71 (*bottom*), which are specific for monocytes-macrophages

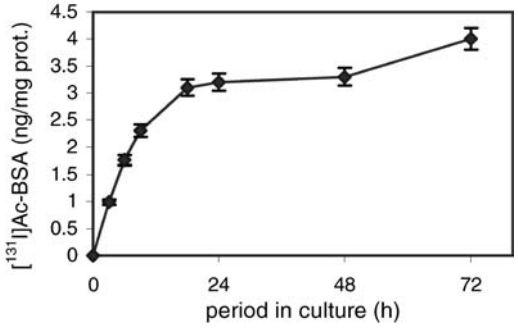


Fig. 2. Endocytosis of [^{131}I]Ac-BSA by monocytes differentiating to macrophages. HMMs (1×10^6/ml) were plated in non-coated plastic dishes at t=0 h and incubated for up to 72 h. After different time intervals, HMMs were washed and medium containing 5 µg/ml [^{131}I]Ac-BSA (700 Bq/µg) was added. The uptake of labelled protein was measured after incubation for 4 h at 37°C as described in Materials and methods. Values are mean±SEM of one typical experiment performed in triplicate

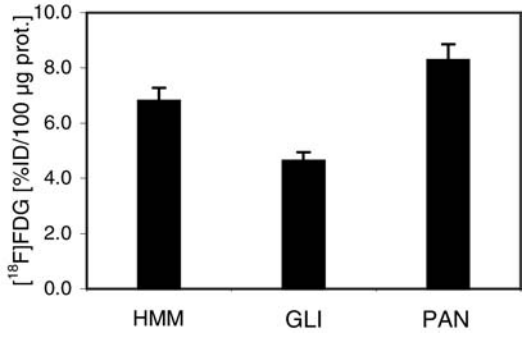


Fig. 3. [^{18}F]FDG uptake in human monocytes differentiated to macrophages (*HMM*), human glioblastoma cells (*GLI*) and human pancreatic adenocarcinoma cells (*PAN*). HMMs differentiated to macrophages during 14 days' incubation, as described in Materials and methods. Incubation with [^{18}F]FDG was performed for 3 h. [^{18}F]FDG uptake is expressed as % ID/100 µg total cell protein to compare proliferative and non-proliferative cells on the same basis. Differences between HMM and tumour cells were statistically significant (P<0.003 and P<0.002)

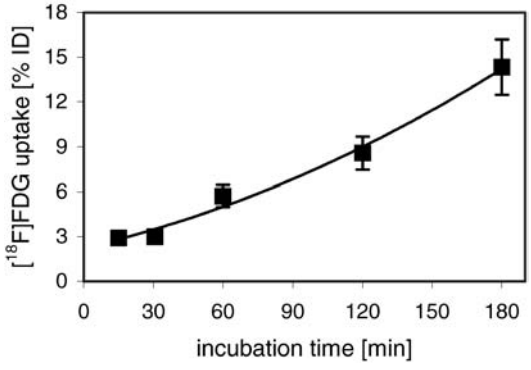


Fig. 4. Time course of [^{18}F]FDG accumulation in human monocytes differentiated to macrophages. HMMs differentiated to macrophages during 14 days' culture as described in Materials and methods. [^{18}F]FDG uptake is expressed as % ID. Values are mean±SEM of four independent determinations within a single experiment. Two similar experiments yielded qualitatively identical results

below 1%, indicating no significant content of granulocytes in our experimental setup.

[^{18}F]FDG uptake in HMMs was in the range of the uptake values measured in human pancreatic carcinoma cells and human glioblastoma cells. Compared with an uptake value of 6.8%±0.34% ID/100 µg protein determined in HMMs, [^{18}F]FDG uptake was slightly higher in human pancreatic cells (8.2%±0.43% ID/100 µg protein), but lower in glioblastoma cells (4.63%±0.32% ID/100 µg protein) (Fig. 3). These differences were significant (n=5, P<0.05).

The uptake of [^{18}F]FDG increased linearly with time. In comparison with an uptake value of 2.92%±0.12% ID after incubation for 15 min, [^{18}F]FDG uptake in HMMs was increased by a factor of 5 after incubation for 3 h (14.34%±1.43% ID, Fig. 4).

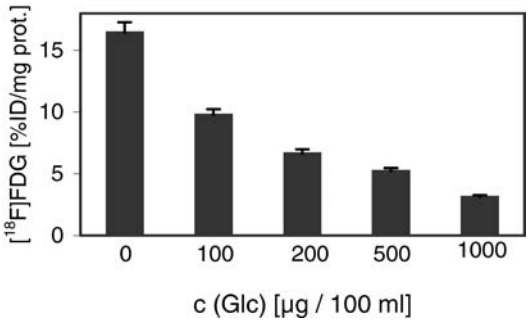


Fig. 5. HMMs were incubated for 3 h with [18F]FDG as described in Materials and methods. Glucose was added in varying concentrations to simulate hyperglycaemic or hypoglycaemic conditions. [18F]FDG uptake decreased with increasing glucose concentrations. Values are mean±SEM of one typical experiment. Two similar experiments yielded qualitatively identical results

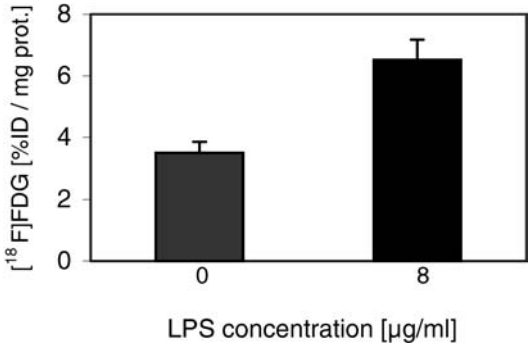


Fig. 7. HMMs were activated with LPS during pre-incubation for 24 h as described in Materials and methods. Incubation with [18F]FDG was performed for 1 h. LPS-activated monocyte-macrophages showed significantly increased [18F]FDG uptake (P<0.005). Values are mean±SEM of a single experiment. Three similar experiments yielded qualitatively identical results

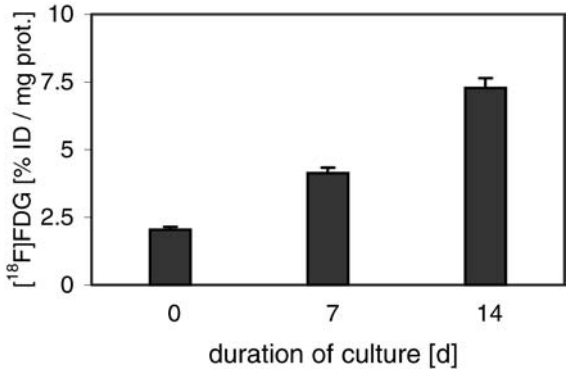


Fig. 6. Incubation with [18F]FDG was performed for 2 h as described in Materials and methods. The duration of cell culture after HMM preparation and plating was varied as indicated. During this period of cell culture, monocytes differentiated to macrophages. [18F]FDG uptake increased significantly with duration of cell culture (P<0.001, n=12)

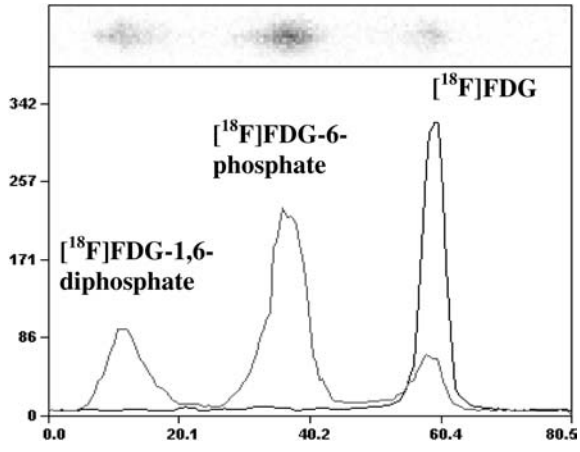


Fig. 8. Radio-thin layer chromatography of intracellular metabolites of [18F]FDG in HMMs. Incubation with [18F]FDG was performed for 3 h as described in Materials and methods. Radio-thin layer chromatography of cellular lysates shows two radioactive intracellular metabolites (*grey line*). Distribution of intracellular radioactive compounds was as follows: [18F]FDG-1,6-diphosphate/[18F]FDG-6-phosphate/[18F]FDG =26:60:14 (% sum of regions). Both radioactive intracellular metabolites can be hydrolysed to [18F]FDG using $HClO_4$ (*black line*)

Experiments to address [18F]FDG release did not reveal relevant [18F]FDG activity after different incubation times. In all cases [18F]FDG activity in the chase medium was below 0.001% ID (data not shown here).

We examined whether the uptake of [18F]FDG depended on glucose concentration in the uptake medium. The concentration of cold glucose in the medium varied between 0 mg/dl and 1,000 mg/dl. [18F]FDG uptake was highest without added glucose (16.44%±1.12% ID) and decreased to 3.1%±0.12% ID at a concentration of cold glucose of 10 mg/ml (Fig. 5).

[18F]FDG uptake increased significantly with cell culture duration. Seven days after plating, HMMs showed twofold higher uptake (4.13%±0.42% ID) than directly after HMM isolation and purification (2.05%±0.2% ID). Fourteen days after plating, HMMs exhibited almost threefold higher [18F]FDG uptake (7.27%±0.52% ID, Fig. 6).

[18F]FDG uptake was increased in LPS-activated HMMs (6.53%±0.92% ID) compared to HMMs without LPS activation (3.51%±0.52% ID). This difference was significant (n=16, P<0.005; Fig. 7).

In order to investigate the intracellular phosphorylation of [18F]FDG radio-thin layer chromatography of cell lysates was performed. [18F]FDG-6-phosphate was detected intracellularly, as shown in Fig. 8. However, a second radioactive metabolite was also detected. This peak represents [18F]FDG-1,6-diphosphate, as shown by radio-high-performance liquid chromatography previously [15]. The distribution of radioactive intracellular compounds was determined to be: [18F]FDG-1,6-diphos-

phate/[^{18}F]FDG-6-phosphate/[^{18}F]FDG =26:60:14 (% sum of regions). In addition, both radioactive metabolites could be hydrolysed to [^{18}F]FDG by the use of perchloric acid ($HClO_4$) (Fig. 8).

Discussion

Besides granulocytes and lymphocytes, macrophages differentiating from blood monocytes are important cellular constituents not only of inflammatory lesions but also of tumours [3, 16]. Therefore, investigation of the factors governing [^{18}F]FDG uptake in human monocytes-macrophages may allow further insight into the mechanisms underlying [^{18}F]FDG accumulation in inflammation and tumours.

The HMM preparation used in this study is a well-established experimental setup for the investigation of macrophages in vitro [12, 17]. In particular, it takes into account the fact that peripheral blood monocytes undergo a process of differentiation to macrophages in vitro [17]. During differentiation, the [^{18}F]FDG uptake by our HMM preparations significantly increased by a factor of approximately 3 and was further enhanced by a factor of 2 by LPS. This effect is due to the fact that phagocytes, neutrophils and also macrophages increase oxidative metabolism when exposed to phagocytable particles or membrane-perturbing agents, a phenomenon termed "respiratory burst" [8, 9, 18, 19]. However, according to Jones et al., [^{3}H]deoxyglucose uptake and respiratory burst activity are temporarily dissociated in granulocytes [9], and this might apply to macrophages as well. A study of the metabolic response of macrophages to different stimuli would help to define the exact relationship between respiratory burst and glucose metabolism in this type of inflammatory cell.

Theoretically, the intracellular [^{18}F]FDG accumulation in phagocytes could be mediated by two different mechanisms: transport via specific transport proteins, as in most other cells, or endocytosis. [^{18}F]FDG accumulated as a consequence of [^{18}F]FDG phosphorylation in our HMM preparations. Furthermore, [^{18}F]FDG accumulation in HMMs was competitively inhibited by increasing glucose concentration in the medium, suggesting its transport into HMMs via specific glucose transport proteins. This result corresponds to the recent finding that GLUT-3 is the predominant transporter on the mouse macrophage cell line RAW 264.7 [20]. The dependence of [^{18}F]FDG uptake on glucose concentration has also been reported in experimental models of inflammation [21]. Nevertheless, Zhuang et al. could not demonstrate this effect using freshly prepared peripheral blood mononuclear cells [22]. In contrast to Zhuang et al., we studied the effect of glucose concentration on [^{18}F]FDG uptake in differentiated and LPS-activated macrophages, which we assume to be more representative for mononuclear cells contained in inflammatory lesions.

Interestingly, radio-thin layer chromatography of our cell lysates disclosed not only [^{18}F]FDG-6-phosphate but also [^{18}F]FDG-1,6-diphosphate as a metabolite of [^{18}F]FDG in HMMs after incubation for 4 h. This is explicable by the little known and as yet not systematically studied fact that [^{18}F]FDG-6-phosphate is a substrate of phosphoglucomutase [23] and glucose 6-phosphatase. Although FDG metabolism beyond its 6-phosphate has no direct clinical implications for PET imaging as long as analyses are performed sufficiently early to prevent further metabolic compartments [24], we focussed on the cellular retention of the detected metabolite [^{18}F]FDG-1,6-diphosphate in vitro. In this study we demonstrated intracellular trapping of both [^{18}F]FDG-6-phosphate and [^{18}F]FDG-1,6-diphosphate in HMMs by performing release experiments.

Our in vitro results support data from previous clinical and experimental studies reporting markedly increased uptake of [^{18}F]FDG at the site of infections and in atherosclerotic large arteries, which has been attributed to activated macrophages and granulocytes [5, 7, 25, 26, 27]. Generally, we found the in vitro model of isolated HMMs to be of good value in predicting the uptake characteristics of [^{18}F]FDG in inflammatory cells, even though we could not verify decreasing [^{18}F]FDG uptake over time as described by Zhuang et al. for inflammatory processes in vivo [28]. The in vitro model described herein defined useful parameters for cell differentiation and activation status; however, in vivo factors that also influence [^{18}F]FDG uptake, e.g. blood supply, lesion architecture or cellular activation status, were naturally not considered.

The content of macrophages in malignant tumours is described to range from 19% to 64% [29]. When averaging the data obtained in different neoplasms, it can be assumed that about 10%–30% of all cellular elements within an untreated human tumour are macrophages [22, 29]. Tracer uptake measured by PET represents the sum of extracellular radioactivity and tracer uptake within all individual cells contained in the region of interest under study. Therefore, FDG uptake by HMMs may also contribute to the overall value of [^{18}F]FDG uptake in neoplastic lesions as determined by PET. We found that [^{18}F]FDG uptake by mature HMMs was in the range of uptake by the two tumour cell lines studied (human primary pancreatic adenocarcinoma cells: BxPC-3; human glioblastoma cells: U-138 MG) when [^{18}F]FDG uptake was quantified as % ID in relation to total protein mass. Extrapolating these in vitro data, between 10% and 30% of the [^{18}F]FDG uptake values measured by PET in neoplastic lesions should be attributed to macrophages.

In contrast to our in vitro results, recent evidence in untreated human breast cancer and non-small cell lung cancer does not indicate that the contribution of macrophages is that significant [30, 31]. However, the density of macrophages in the tumours studied by Brown et al. was rather low, i.e. 8% of all cellular elements, and gen-

eralisation of these findings to other types of tumour can therefore only be performed with caution. Bos et al. semiquantitatively evaluated the density of macrophages in immunohistochemical specimens of untreated breast cancers. In approximately 50% of their cases the occurrence of macrophages was scored as frequent, so that their results contrast with what one would expect when extrapolating our in vitro data. A possible, but speculative, explanation is that macrophages contained in tumours are not as active as those prevailing in infection.

Other studies support our assumption that the contribution of macrophages is significant for the overall [^{18}F]FDG uptake by neoplastic lesions. Data reported by Kubota et al. demonstrated that 29% of [^{18}F]FDG uptake in tumours was related to macrophages [3]. In their animal model, the density of macrophages and also [^{18}F]FDG uptake was particularly high adjacent to tumour necrosis. This suggests that the contribution of inflammatory cells to the [^{18}F]FDG uptake values in tumours is of particular importance in neoplasms containing necrotic areas induced by, for example, chemotherapy. In this context, it is interesting that, compared with other tracers such as radiolabelled amino acids, [^{18}F]FDG-PET has shortcomings for monitoring the response of tumours to treatment [32]. There are various possible explanations for the rather low sensitivity of [^{18}F]FDG-PET in monitoring response to chemotherapy; besides the phenomenon of increased [^{18}F]FDG uptake in cells under stress [33], the presence of a larger number of active inflammatory cells removing necrotic areas may also increase the overall [^{18}F]FDG uptake within a treated tumour.

The data presented here on [^{18}F]FDG uptake in HMMs provides the basis for a suitable cell model for the evaluation of the accumulation in macrophages of other PET radiopharmaceuticals, such as ^{18}F-labelled amino acids. Clearly, cultures of other inflammatory cells such as neutrophils or lymphocytes, as presented recently [8, 9], would be helpful in enabling further comparisons to be made.

In conclusion: We determined [^{18}F]FDG uptake in inflammatory cells by applying a well-defined cell model of isolated human macrophages in vitro. Our findings clearly indicate that [^{18}F]FDG uptake values are in the range measured in tumour cell lines (human glioblastoma and pancreatic carcinoma cells). [^{18}F]FDG uptake is specifically influenced by macrophage differentiation and cell activation. These data acquired using our cell model of isolated human macrophages could have important implications for the analysis of [^{18}F]FDG uptake in vivo by PET in inflammatory and neoplastic lesions.

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