18F-CPFPX PET Identifies Changes in Cerebral A1 Adenosine Receptor Density Caused by Glioma Invasion

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Adenosine plays a critical role in both tumor proliferation and the cerebral response to tumor invasion. We used 8-cyclopentyl-3-(3-18F-fluoropropyl)-1-propylxanthine (18F-CPFPX) PET to investigate A1 adenosine receptor (A1AR) density as a potential indicator of the local cerebral response to glioma invasion. 

Methods: A1AR density in F98 glioma–bearing rats was examined by 18F-CPFPX and 3H-CPFPX using PET, quantitative in vitro and ex vivo double-label receptor autoradiography, and immunohistochemical analyses. Results: For all imaging modalities, A1AR signal intensity was increased in a zone surrounding experimental tumors (136%–146% that in control tissue) (P < 0.01). Immunostaining identified activated astrocytes as the main origin of peritumoral A1AR upregulation. The results of a pilot 18F-CPFPX PET study on a patient with recurrent glioblastoma multiforme confirmed increases in A1AR density in the immediate vicinity of the tumor. Conclusion: 18F-CPFPX PET is suitable for the detection of peritumoral changes in A1AR density. Molecular imaging with 18F-CPFPX PET may open novel possibilities for gaining experimental and clinical insights into the cerebral response to tumor invasion.

Key Words: brain tumor; A1 adenosine receptor; tumor invasion; 18F-CPFPX; molecular imaging


Neoplasms arising from glial cells are the most frequent primary brain tumors in adults. Despite considerable efforts, these tumors remain almost incurable because of their highly proliferative, infiltrative, and aggressive behaviors (1). The modulation of appropriate receptors in the invasion zone of tumors could augment cerebral antitumor responses, thus serving as an adjunct to tumor therapy. Identification and monitoring of peritumoral changes indicating the type and extent of the cerebral response to tumor invasion could advance this increasingly important branch of experimental and clinical research on brain tumors.

Adenosine is a ubiquitous modulator of cell metabolism and plays an important role in tumor growth and diffusion (2). It can promote tumor survival by stimulating carcinoma proliferation (3), and it is able to suppress the local antitumor immune response (4), which is partly mediated by adenosine surface receptors. Adenosine receptors expressed by cells in the invasion zone of tumors therefore may be targets for therapy strategies intended to augment antitumor responses and to block tumor proliferation. In this study, we investigate whether the novel A1 adenosine receptor (A1AR) PET ligand 8-cyclopentyl-3-(3-18F-fluoropropyl)-1-propylxanthine (18F-CPFPX) (5) is suitable for the detection of peritumoral A1ARs, which may serve as indicators of the cerebral response to tumor invasion.

MATERIALS AND METHODS

Cell Culture and Tumor Implantation

A previous publication (6) described the cell culture and the technique of tumor implantation in detail. The care and use of animals conformed to German law and received institutional approval (23.203.2 KFA 6/98). For tumor cell implantation, 13 male CDF (F-344) Fischer rats (body weight, 230–250 g; 8–12 wk old; Charles River Laboratories) were used. A suspension of F98 cells (either 104 or 106 cells in 5 µL saline) was stereotactically injected at a depth of 6.5 mm from the dural surface approximately 2 mm posterior and 4 mm lateral to the bregma. Neither intra- nor postoperative complications occurred. All experiments were performed 10 d after F98 cell implantation.

Immunohistochemical Analyses

At 7 d after F98 cell implantation, 5 rats were anesthetized with pentobarbital and transcardially perfused with 30 mL of physiologic saline and then with 120 mL of saline containing 20% sucrose for cryopreservation. For immunohistochemical studies, slices (20 µm) were submerged in Zamboni’s fixative for 48 h and then in 25% sucrose:phosphate-buffered saline (PBS; sodium phosphate at 10 mmol/L in 0.9% NaCl; pH 7.4), frozen, and sectioned at 50 µm. After 4 rinses in PBS, freely floating sections
were incubated for 1 h in PBS containing 0.1% Triton X-100 and 10% normal goat serum (Vectastain; Camon). The sections then were incubated at 4°C for 48 h with primary monoclonal antibody to glial fibrillary acid protein (GFAP) (1:25; Boehringer) and polyclonal antibody to A1AR (1:300; Sigma). Alternate sections were immunostained with primary antibody to GFAP or microglia marker OX-42 (1:100; Serotec) and processed as previously described (7).

**Radiopharmaceuticals**

$^3$H-CPFPX (8), $^{18}$F-CPFPX (9), and O-(2-$^{18}$F-fluoroethyl)-L-tyrosine ($^{18}$F-FET) (10) were synthesized with specific radioactivities of $\geq 2.1$, $\geq 270$, and $\geq 20$ TBq/mmol, respectively, and a radiochemical purity exceeding 98%. They were dissolved in 0.9% saline containing 10% ethanol for intravenous injection and administered in PBS (pH 7.4).

**Autoradiography and Image Processing**

At 7 days after F98 cell implantation, 3 rats received an intravenous injection of a mixture of 10 MBq of $^3$H-CPFPX and 50 MBq of $^{18}$F-CPFPX. Two other rats received a mixture of 10 MBq of $^3$H-CPFPX and 50 MBq of $^{18}$F-FET. Animals were killed 20 min after tracer injection; the brains were removed immediately, frozen in 2-methylbutane at −50°C, and cut in coronal sections (20 µm) (CM 3050 instrument; Leica). Sections were placed on phos- phorimaging plates along with industrial tritium activity standards (Microscales; Amersham Biosciences) for tritium studies and with calibrated $^{18}$F-labeled liver paste standards made in-house. The distribution of $^{18}$F radioactivity was recorded on imaging plates insensitive to β-particles of tritium (BAS-SR 2025; Raytest-Fuji). Tritium distribution was detected after the decay of $^{18}$F (10 half-lives) by use of a high-performance imaging plate reader (BAS5000 BioImage Analyzer; Raytest-Fuji) and tritium-sensitive imaging plates (BAS-TR 2025; Raytest-Fuji).

For in vitro displacement studies, brain sections were taken from 5 animals from the ex vivo studies and incubated at 22°C for 120 min with $^3$H-CPFPX at 4.4 nM, with or without $R$-N6-(2-phenylisopropyl)adenosine at 100 µM, in Tris:HCl buffer (170 mM, pH 7.4) containing adenosine deaminase at 2 IU/L and 5′-guanylimidodiphosphate at 100 µM. An irregular region-of-interest analysis was carried out by standard image analysis (AIDA 2.31; Raytest), and statistical software (Prism 4.00; GraphPad) was used to process the digitized autoradiographic data.

**High-Resolution Small-Animal PET and MRI**

Three animals underwent sequential PET and MRI. Previous publications (11,12) described the experimental setup, data acquisition, and creation of high-resolution PET images. Animals were anesthetized with ketamine (100 mg/kg); xylazine (10 mg/kg) and received a bolus injection of 0.3 mL of $^{18}$F-CPFPX (37 MBq/mL dissolved in 0.9% NaCl:7% ethanol). PET data were acquired for 60 min after injection in 10 frames, each with a duration of 6 min. Upon completion of the PET scanning, the animals were killed by cervical dislocation, and their brains were processed for autoradiography.

**Human PET Investigation**

The human PET measurement method was approved by the Ethics Committee of the Medical Faculty of Heinrich-Heine-University Düsseldorf and was performed in accordance with a recently described method (5) after written informed consent had been obtained from the patient. In brief, the PET study was performed in three-dimensional mode with an ECAT EXACT HR+ scanner (Siemens-CTI). A slow bolus of 214.1 MBq (5.79 mCi) of $^{18}$F-CPFPX in 10 mL of saline was injected intravenously. Dynamic data were acquired over a 60-min period after injection. PET data were corrected for random and scattered radiation as well as attenuation. Data were subsequently Fourier rebinned into two-dimensional sinograms and reconstructed by filtered backprojection (Shepp filter, 2.5 mm) with a pixel size of 2 x 2 mm$^2$. The ratio of specific uptake to nonspecific uptake, represented by (region-of-interest uptake − cerebellar uptake)/cerebellar uptake, was calculated as a surrogate of the regional binding potential.

**RESULTS**

We observed a significant increase in $^3$H-CPFPX binding in the vicinity of implanted F98 gliomas (Figs. 1A and 1B). Peritumoral A1AR density ranged between 54.1 and 100.1 fmol/mg (wet weight), a level 136%–146% that in control tissue (contralateral temporal cortex) and 168%–198% that in the total tumor. Average absolute peritumoral A1AR-binding capacity was significantly higher than receptor binding in the tumor ($P < 0.01$) and in the control region ($P < 0.01$). Outside the tumor, the pattern of $^3$H-CPFPX binding was typical of that of rat cerebral A1ARs, that is, low nonspecific background binding (maximum 7% total binding) and high specific binding in the cerebral cortex, cerebellar cortex, hippocampus, and distinct thalamic nuclei (12). In vitro autoradiography with $^{18}$F-CPFPX excluded the possibility that peritumoral tracer accumulation was caused primarily by pathologically increased permeability of the blood–brain barrier. Ex vivo and in vitro patterns of peritumoral A1AR binding were identical. $R$-N6-(2-phenylisopropyl)adenosine at 100 µM completely displaced $^3$H-CPFPX binding, demonstrating that the radioligand is bound specifically to A1ARs.

Double-label autoradiography with $^3$H-CPFPX and $^{18}$F-FET was used to determine whether the zone of increased A1AR density was part of the tumor or located adjacent to it (6). The solid parts of tumors exhibited homogeneous $^{18}$F-FET uptake (Fig. 1C). Coregistration of $^3$H-CPFPX and $^{18}$F-FET autoradiographs showed that the region of increased A1AR density was located primarily outside the tumor mass, slightly overlapping the solid parts of tumors (Fig. 1D). The histologic findings (Fig. 1E) confirmed that conclusion.

Cell types responsible for the increase in A1AR density were identified by immunohistochemical analyses. GFAP, OX-42, and a nonphosphorylated neurofilament (SMI311) served as markers for astrocytes, activated microglia, and neurons, respectively. GFAP immunoreactivity was enhanced in the vicinity of tumors, matching the distribution of A1ARs (Fig. 2A). In contrast, the zone of activated microglia, defined by OX-42 immunoreactivity, extended far beyond the vicinity of tumors (Fig. 2B). Double-label immunofluorescence revealed a partial overlap of A1AR immunoreactivity and GFAP immunoreactivity (Figs. 2C and 2D). In the vicinity of tumors, colocalization of A1AR...
labeling and SMI311 immunoreactivity was seen (Fig. 2E). In accordance with the autoradiographic findings for $A_1$AR expression, several tumor cells also showed $A_1$AR immunoreactivity (Fig. 2C).

FIGURE 1. Autoradiographic studies. (A) Quantification of data from F98 glioma ex vivo animal experiments reveals that receptor densities in tumor margin, tumor (total), and control region (contralateral temporal cortex [Temp. ctx.]) differ significantly ($P < 0.01$) (asterisks). WW = wet weight. Error bars indicate SDs. (B) Two representative ex vivo autoradiographs display intense accumulation of $^3$H-CPFPX at periphery of tumor. Left panel displays solid tumor; right panel displays tumor with central necrosis. Ex vivo experiments were performed 10 d after implantation of F98 tumor cells in right caudate putamen. (C) Same sections as in B. Ex vivo autoradiographs obtained with $^{18}$F-FET delineate solid tumor mass. For direct reading of absolute receptor density and amino acid (AA) uptake, see black–white scales between sections. (D) Histograms of $^3$H-CPFPX (red line), $^{18}$F-FET (green line), and Nissl stain (blue line) taken from levels indicated by respective colored lines in B, C, and E. $A_1$AR density peaks at margin of solid tumor mass. OD = optical density. (E) Nissl stains of 2 sections depicted in B and C.

FIGURE 2. Immunohistochemical analyses and fluorescence microscopy of sections from rat brain gliomas 10 d after implantation of tumor cells in right caudate putamen. (A) GFAP immunoreactivity of reactive astrocytes is increased in immediate vicinity of tumor (Tu), as indicated by arrowheads. Scale bar = 1 mm. (B) OX-42 immunoreactivity as a marker of activated microglia spreads far beyond peritumoral zone into cortical and limbic regions, as indicated by arrowheads. Scale bar = 1 mm. (C) Confocal fluorescence images of GFAP (green) and $A_1$AR (red) in tumor periphery indicate that a subpopulation of GFAP-immunopositive cells expresses $A_1$ARs (yellow). Tumor cells in solid tumor mass as well as in invasion zone (arrowheads) are also immunoreactive for $A_1$ARs (red). Scale bar = 200 μm. (D) High magnification of C. Subpopulation of astrocytes closely associated with a vessel (V) in tumor margin is immunoreactive for both GFAP (green) and $A_1$ARs (red). Cellular colocalization is indicated by yellow. $A_1$AR immunoreactivity (red) is also found next to cells staining for GFAP. Scale bar = 50 μm. (E) Confocal microscopy of neuronal marker SMI311 (green) and $A_1$AR (red) indicating cellular colocalization of both immunostains (yellow). Scale bar = 50 μm.
Combined high-resolution MRI and A₁AR PET (11,12) confirmed the usefulness of in vivo imaging in the F98 glioma model. MR images served to identify tumors and anatomic structures (Fig. 3A). The MRI tumor contour served as an overlay, which was transferred to individually coregistered animal PET datasets. After intravenous injection of ¹⁸F-CPFPX, brain uptake was rapid (12), reaching the maximum in less than 6 min. A high accumulation of ¹⁸F-CPFPX clearly marked the border of the tumor (Fig. 3B). PET experiments were complemented by ex vivo dis-

**DISCUSSION**

In this study, we investigated peritumoral A₁ARs as potential molecular targets for monitoring the cerebral response to glioma invasion. Reports of increased expression of A₁AR mRNA in colorectal cancer and adjacent mucosa (13) have indicated that cancer-associated alterations in A₁AR density are not restricted to brain tissue. This finding may indicate that a nonspecific factor contributes to A₁AR upregulation. Microdialysis studies of human gliomas with a high grade of malignancy (14) have shown that extracel-

**FIGURE 3.** Representative examples of tumor-bearing rat 10 d after implantation of F98 cells in left caudate putamen, as measured by ¹⁸F-CPFPX and high-resolution small-animal PET. (A) MRI depicting tumor (red arrows) as hypointense zone in coronal (left) and horizontal (right) sections. (B) Summed PET images of coronal (left) and horizontal (right) sections (frames 3–10, 48-min scan time). White tracing outlines outer brain contour based on individually coregistered MRI datasets depicted in A, which were subsequently superimposed onto PET datasets. Note significant tracer accumulation at tumor border (red arrows), coinciding with outer border of hypointense zone determined by MRI (red arrows in A). Other signals represent normal cerebral tracer accumulation and the tracer and its metabolites in blood vessels. (C) Ex vivo autoradiograph (left) taken from PET experiment shown in B confirms circumferential accumulation of ¹⁸F-CPFPX at the tumor margin (Fig. 3C).

A PET study of a 43-y-old patient with histologically proven recurrent glioblastoma multiforme revealed a high accumulation of ¹⁸F-CPFPX at the circumference of the tumor and a lower accumulation in the center of the tumor (Fig. 4), corresponding to the findings from the experimental F98 glioma model.

**FIGURE 4.** Human PET study. (A) Coregistered planes of contrast agent–enhanced MR image (left) and ¹⁸F-CPFPX PET image (right) of recurrent glioblastoma multiforme in right tempor-oral region. Circular zone (white arrowheads) in MR image indicates top of solid tumor and surrounding tissue. (B) Circular accumulation (boxed area) of ¹⁸F-CPFPX (right) corresponding to contrast agent–enhanced region in MR image (left). (C) High magnification of B. Adjacent to circular peritumoral ligand accumulation, a zone of gray matter, most likely temporal cortex, has been compressed and displaced by tumor (arrowheads).
lular adenosine levels are significantly lower in tumors than in control tissue. Thus, chronically low levels of endogenous adenosine may induce the upregulation of A₁ARs on cells at the tumor periphery.

Besides nonspecific effects, changes in the cellular composition of the invasion zone may be responsible for increased A₁AR density. Peritumoral tissue is invaded by tumor and vascular endothelial cells and infiltrated by activated macrophages, microglia, and reactive astrocytes. Our autoradiographic and immunohistochemical studies demonstrated that increased peritumoral A₁AR density occurs mainly on GFAP-positive astrocytes. Activated astrocytes contribute significantly to tissue repair in the zone surrounding brain tumors as well as ischemic infarcts and trauma (15,16). Consistent with such a role, A₁AR density is also increased at the borders of central necroses in experimental gliomas.

Besides its potential role in the local cerebral response to tumor invasion, a change in cerebral A₁AR density may have implications for tumor-associated epilepsy. Adenosine, acting through both pre- and postsynaptic A₁ARs, is a highly potent antictal neuromodulator (17). Because epilepsy occurs frequently in cerebral tumors (18), A₁ARs at tumor borders may represent potential targets for the treatment of this type of epilepsy.

Investigating the potential diagnostic and therapeutic consequences of peritumoral A₁AR upregulation will inevitably require in vivo studies, because the complexity of interactions between the tumor and the reactive zone can hardly be emulated in vitro. We demonstrate that A₁ARs in the peritumoral zone can be identified in vivo by ¹⁸F-CPPFX PET in both experimental rat brain tumors and human gliomas. Thus, molecular imaging with ¹⁸F-CPPFX PET will both allow longitudinal animal studies of pharmaceutical modulation of peritumoral A₁ARs and permit clinical investigations to collect data on tumor invasion in humans.

CONCLUSION

In this study, we investigated cerebral A₁ARs as potential indicators of the cerebral response to glioma invasion. For all imaging modalities, A₁AR signal intensity was increased in a zone surrounding experimental tumors in a rat glioma model. The results of the first ¹⁸F-CPPFX PET study on a patient with recurrent glioblastoma confirmed the findings of animal experiments indicating that A₁AR participates in the cerebral response to tumor invasion. Molecular imaging with ¹⁸F-CPPFX PET thus may open novel possibilities for gaining experimental and clinical insights into the cerebral response to tumor invasion.

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REFERENCES