A1 Adenosine Receptors in Microglia Control Glioblastoma-Host Interaction

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Abstract

We report that experimental glioblastoma grow more vigorously in A1 adenosine receptor (A1AR)–deficient mice associated with a strong accumulation of microglial cells at and around the tumors. A1ARs were prominently expressed in microglia associated with tumor cells as revealed with immunocytochemistry but low in microglia in the unaffected brain tissue. The A1AR could also be detected on microglia from human glioblastoma resections. To study functional interactions between tumor and host cells, we studied glioblastoma growth in organotypical brain slice cultures. A1AR agonists suppressed tumor growth. When, however, microglial cells were depleted from the slices, the agonists even stimulated tumor growth. Thus, adenosine attenuates glioblastoma growth acting via A1AR in microglia. (Cancer Res 2006; 66(17): 8550-7)

Introduction

Adenosine is a regulatory nucleoside that is generated in response to cellular stress and damage and is therefore increased during episodes of tissue hypoxia and inflammation. Adenosine is a ubiquitous biological mediator with the capacity to produce both proinflammatory and anti-inflammatory effects in tissues and acts through four distinct cell membrane receptors (A1AR, A2AAR, A2BAR, and A3AR) each with varying ligand affinities, tissue distributions, and signal transduction mechanisms. The A1AR has a high affinity for adenosine and has been implicated in both proinflammatory and anti-inflammatory aspects of disease processes. On one side, A1AR signaling can promote neutrophil (1) and monocyte (2, 3) activation, whereas, on the other side, A1AR signaling is involved in anti-inflammatory and protective pathways in neuroinflammation and injury (4) and in cardiac (5) and renal (6, 7) injury. Adenosine-mediated anti-inflammatory effects have been studied extensively in macrophages and macrophage cell lines. Adenosine inhibits the production of several proinflammatory cytokines [transforming growth factor-α, interleukin (IL)-6, and IL-8] by lipopolysaccharide-stimulated macrophages and enhances the release of the anti-inflammatory cytokine IL-10 (8–10). Recent studies suggest an anti-inflammatory role for chronic A1AR activation by high levels of adenosine in the lung, a surprising and important finding in light of the fact that A1AR antagonists are being investigated as a potential treatment for asthma (11).

In the central nervous system (CNS), the A1AR is highly expressed on microglia/macrophages and neurons (12). In the latter, A1AR is coupled to activation of K+ channels (13) and inhibition of Ca2+ channels (14), both mechanisms that attenuate neuronal excitability. By reducing excitotoxicity, adenosine can act as a neuroprotective factor. Because A1 adenosine receptors (A1AR) are expressed throughout the brain (15), adenosine has the potential to be involved in different brain pathologies. Although A1ARs modulate physiologic functions, A1AR-deficient mice show no obvious abnormal behavior (16, 17). However, exposed to pathophysiologic conditions, such as hypoxia, A1AR-deficient mice show more neuronal damage and have a lower survival rate. It was therefore concluded that A1ARs are primarily important in mediating effects of adenosine during pathophysiologic conditions (16, 17). In the present study, we have addressed the question whether A1ARs are involved in another brain disease (i.e., the development of primary brain tumors). We have, in particular, focused on the interaction of the tumor cells with the intrinsic immune cells of the brain, the microglial cells.

Materials and Methods

Animals. Animals were Fisher CD344 rats (Charles River Breeding Laboratories, Schöneiche, Germany), wild-type C57BL/6 (Charles River Breeding Laboratories), NMRI mice, nestin-green fluorescent protein (GFP) mice (genetic background of C57BL/6; bred and genotyped as described previously; refs. 18, 19), glial fibrillary acidic protein (GFAP)-GFP mice (genetic background of C57BL/6; bred and genotyped as described previously by our group; ref. 20), CX3CR1-GFP mice [genetic background of SV129/C57BL/6; bred and genotyped as described by Jung et al. (21)], and homozygous A1AR-null mice (A1AR−/−) and littermate wild-type (A1AR+/−) controls [both with SV129/C57BL/6; bred as described previously by Sun et al. (22)]. Briefly, heterozygous mice were bred to obtain homozygous A1AR-deficient mice and wild-type littermates. Animals were genotyped after performing PCR on samples from tail cuts. Message for the A1AR was amplified by the following primers: A1AR sense 5’-GTACATCTCGCCCTGCCTGGCAAG-3’ and antisense 5’-GAGAATACCTGGCCTGCCTGGCAAG-3’. Glucose transporter (GluKAT) G3261 (isogenic to C57BL/6; mice; National Cancer Institute-Frederick, Frederick, MD) and F98 glioblastoma cells (isogenic to Fisher CD344 rats; American Type Culture Collection, Manassas, VA) were grown in DME (Invitrogen, Heidelberg, Germany) with additives.

Microglial cell cultures. Microglial cultures were prepared from cerebral cortex of newborn C57BL/6 mice as described previously (23).
In brief, the brain was carefully freed of blood vessels and meninges. Cortical tissue was trypsinized for 2 minutes, dissociated with a fire-polished pipette, and washed twice. Mixed glial cells were cultured for 9 to 12 days in DMEM supplemented with 10% FCS, 2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin with medium changes every third day. Microglial cells were then separated from the underlying astrocytic layer by gentle shaking of the flask for 1 hour at 37°C in a shaker-incubator (100 rpm). The cells were seeded in six-well plates at a density 10^5/well. Cultures usually contained >95% microglial cells, which could be stained with Griffonia simplicifolia isolecitin B4 (Sigma-Aldrich, Deisenhofen, Germany), a marker for microglia. Cultures were used for experiments 1 to 5 days after plating. Cell medium and supplements were purchased from Seromed/Biochrom (Berlin, Germany).

**Stable transfection.** Gl261 and F98 glioblastoma cells were either transfected with the pEGFP-N1 vector or with the pdsred2-N1 vector both purchased from Clontech (Heidelberg, Germany) using LipofectAMINE 2000 transfection according to the manufacturer's protocols (Invitrogen).

**Immunofluorescence.** Tissue from six animals was used per immunohistochemical experiment (i.e., for each immunohistochemical marker). Three of these animals belonged to the control group (control treatment or wild-type genetic background) and three mice were either treated with pharmacologic compounds or had a deletion in the gene coding for the A1,AR. All staining was done on 40-μm free-floating sections and immunofluorescent triple labeling was done as described previously (19) with the following primary antibodies: polyclonal mouse, rat, and human anti-A,AR (Chemicon, Temecula, CA); guinea pig anti-GAP (Advanced Immunochemical, Long Beach, CA); rabbit anti-GFP (Abcam, Cambridge-shire, United Kingdom); goat anti-GFP (DPC, Bad Nauheim, Germany); mouse anti-dsred (Abcam); rabbit anti-S100β (Swaet, Bellinzona, Switzerland); mouse anti-rat CD11b (Serotec, Oxford, United Kingdom); monoclonal mouse anti-O4 (Chemicon); and polyclonal mouse anti-Iba-1 (Chemicon). FITC-, rhodamine X-, or Cy5-conjugated were used as secondary antibodies (all from Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescent sections were mounted in polyvinyl alcohol with diazabicocytocin as an antifading agent.

**Confocal microscopy.** All confocal microscopy was done using a spectral confocal microscope (Leica, Nussloch, Germany). Appropriate gain and black level settings were determined on control tissues stained with secondary antibodies alone. Overview images were processed with Photoshop version CS (Adobe, San Jose, CA), and colorization images were processed with Velocity version 2.6.1 (Velocity, Lexington, MA).

**Quantification of Iba-1 immunoreactivity.** In a blinded study, we did cell counts of Iba-1-immunopositive cells. Three randomly chosen fields in the tumor border of the respective staining were analyzed. Quantitative analysis of Iba-1-positive cells per square millimeter was done using Image-Pro version 5 (Media Cybernetics, Silver Spring, MD).

**Organotypical brain slice model.** Organotypical brain slice culture preparation was done as described previously by us (24). Brain tissue was derived from 16-day-old male C57BL/6 mice (Animal Breeding Facility, Schönwalde, Germany). For organotypical brain slice preparations, mice were decapitated and the brain was removed within 2 to 3 minutes and placed in ice-cold (4°C) PBS under sterile conditions. The forebrain was dissected from the brainstem and was glued (cyanoacrylate glue) onto a glass block and cut in the coronal plane into 250-μm sections with a vibratome (VT1000S, Leica Co., Heidelberg, Germany). The brain slices were transferred onto the 0.4-μm polycarbonate membrane in the upper chamber of a Transwell tissue insert (Falcon model 3090, Becton Dickinson, Lincoln Park, NJ), which was inserted into a six-well plate (Falcon model 3002, Becton Dickinson). Thereafter, the brain slices were incubated in 1 mL culture medium per well containing DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biological, Atlanta, GA), 0.2 mmol/L l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (median 1). After overnight equilibration of the brain slices in medium 1, this was exchanged for cultivation medium (medium 2). Medium 2 (100 mL) contained 25 mL heat-inactivated horse serum, 25% of 580 μL bicarbonate (7.5%), 2 mL of L-glutamine solution, 2% of 25 mL HBSS, 2.46 mg/mL of 100 μL insulin (all from Life Technologies), 1.2% glucose (20%, Braun Melsungen AG, Germany), 1 mg/mL of 80 μg vitamin C (Sigma-Aldrich), 0.8 μL/mL of 1 mL penicillin/streptomycin (Sigma-Aldrich), and 500 μL of 1 mL Tris in DMEM. Adenosine and X̂ cyclopentyladenosine (CPA) were added daily.

**Inoculation of GI261 and F98 cells into rats and mice.** Anesthetized CD344 Fisher rats, nestin-GFP, GFAP-GFP, CX3CR1-GFP; A1,AR wild-type, A1,AR knockout, and wild-type C57BL/6 mice were immobilized and mounted into a stereotactic head holder (David Kopf Instruments, Tujunga, CA) in the flat-skull position. Approximately 1.5 mm anterior and 1.5 mm lateral to the bregma a 1 μL of 30-gauge gas-tight syringe (Hamilton, Reno, NV) was then inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface, and 1 μL GFP-transfected F98, GFP-transfected Gl261, or dsred-transfected Gl261 cell suspension (10^5 cells in 1 μL serum-free DMEM) were injected. Animals were examined daily for alertness, focal motor deficit, gait disturbance, and responses to contact. Intraoperative or postoperative complications were not observed.

**Tumor cell injection into organotypical brain slices.** About 10^5 GFP-transfected GI261 tumor cells within a defined injection volume (0.1 μL) were inoculated into the slices using a syringe mounted to a micromanipulator. An injection canal was formed, reaching 150 μm deep into the 250-μm-thick slice. Then, the needle was retracted by 50 μm, leaving an injection cavity of ~50 μm. The cell suspension was slowly injected for 3 minutes; subsequently, the syringe was slowly pulled out in 10-μm incremental steps. To ensure identical experimental conditions, the glioblastomas were always inoculated into the same area. Directly after glioblastoma injection, the tumor cells remained at the inoculation site, which could therefore mark the point of origin for all further movements of these cells. Careful control of the injection procedure ensured that no cells were spilled onto the surface of the slices to avoid migration of cells on the surface rather than through the tissue.

**Preparation of clodronate-filled liposomes.** Clodronate-loaded liposomes were obtained from G0T Therapeutics (Berlin, Germany) and the Department of Molecular Cell Biology, Free University of Amsterdam and were prepared as described previously by us (24). All liposomes were passed through a 12-μm filter immediately before use to eliminate large lipid aggregates (25).

**Intraoperative specimens.** Five specimens of neuropathologically confirmed glioblastomas were obtained during planned neurosurgical excisions. The specimens were frozen in liquid nitrogen immediately after excision and stored at −80°C. Sections were immunostained using the above-mentioned primary and secondary antibodies. Fluorescent sections were mounted in polyvinyl alcohol with diazabicocytocin as antifading agent.

**Quantification of tumor volume.** An examiner who was unaware of the slide identity did quantification of tumor volume according to the Cavalieri principle by determining the tumor area in every sixth 40-μm brain slice and then multiplying by 6 × 40 μm using Image-Pro version 5.

**Gelatin zymography.** Activity of gelatinases [matrix metalloproteinase (MMP)-2] was analyzed with the gelatin zymography procedure adapted from that of Heussen and Dowdle (26). Gl261 cells were grown as described above and conditioned medium was taken after 24 hours of culturing. Cultivated microglia (culture method described above) were exposed to unconditioned medium (controls) or stimulated with the glioma conditioned medium containing either 100 μmol/L adenosine or conditioned medium alone for 6 hours. Subsequently, after a brief wash of the microglia, cultures were maintained for a further 24 hours in serum-free culture medium. These microglia conditioned media were mixed with sample buffer (0.5 mol/L Tris (pH 6.8), 20% glycerol, 4% SDS, 0.1% Triton X-100) and the samples were loaded into 7.5% SDS gel containing 1% gelatin. After electrophoresis (4°C, 90 V), gel was washed 2 × 15 minutes in 2.5% Triton X-100 washing solution and incubated overnight in developing buffer [50 mmol/L Tris (pH 7.6), 10 mmol/L CaCl₂, 50 mmol/L NaCl, 0.05% Brij35]. Next, the gel was stained for 30 minutes in 0.5% Coomassie blue solution and later destained with 40% methanol and 10% acetic acid. Gelatinase activity resulted in clear bands on dark blue background and this activity could be quantified. Gelatinase types were identified by molecular weight markers.
Statistical analysis. Data are expressed as mean ± SD. Results were analyzed by Student’s t test for statistical significance after testing for normal distribution done with SPSS version 11.0 (SPSS, Inc., Chicago, IL). Differences with $P < 0.05$ and $P < 0.01$ were considered significant.

Results

A$_1$AR deficiency in the host brain promotes the growth of inoculated glioblastoma cells. To study the importance of adenosine receptors for tumor growth in the central nervous system, we inoculated tumor cells into A$_1$AR$^{-/-}$ mice and A$_1$AR$^{+/+}$ littermate controls. With this approach, we deleted the A$_1$AR in the host cells but not in the inoculated Gl261 glioblastoma cells. Animals were sacrificed 14 days after Gl261 inoculation and the tumor volume was determined double-blinded in axial section according to the Cavalieri principle. The tumor volume in A$_1$AR$^{-/-}$ mice was significantly larger compared with A$_1$AR$^{+/+}$ mice [mean ± SD, 2.96 ± 0.48 mm$^3$ for control ($n = 14$) and 5.21 ± 0.53 mm$^3$ for A$_1$AR$^{-/-}$ mice ($n = 12$); $P < 0.01$; Fig. 1B]. There were no differences in neurologic symptoms (levels of alertness, behavior, and appearance of focal neurologic deficits; e.g., epileptic fits or pareses) between the groups within the 14 days after Gl261 inoculation.

To analyze the cell populations from the host in the vicinity of the tumor cells, we studied the distribution of microglial cells and astrocytes in A$_1$AR$^{-/-}$ and A$_1$AR$^{+/+}$ mice. Immunoreactivity for the macrophage/microglia marker Iba-1 revealed an accumulation of Iba-1-positive cells at the tumor border (Fig. 1C and D). In A$_1$AR$^{-/-}$ mice, the density and number of Iba-1-positive cells was significantly higher compared with wild-type littermates [mean ± SD, 291 ± 59 cells/mm$^2$ for A$_1$AR$^{+/+}$ ($n = 15$) and 547 ± 123 cells/mm$^2$ for A$_1$AR$^{-/-}$ mice ($n = 15$); $P < 0.01$; Fig. 1C]. No differences in the GFAP-positive cell population was observed comparing A$_1$AR$^{-/-}$ and A$_1$AR$^{+/+}$ (data not shown). These results imply that A$_1$AR modulate tumor growth and that microglial cells are the cellular candidates to mediate this effect.

Figure 1. GI261 glioblastoma growth in A$_1$AR$^{+/+}$ and A$_1$AR$^{-/-}$ animals. A, phase-contrast images corresponding to confocal images in (B-D) displaying the brain morphology 14 days after tumor inoculation into a A$_1$AR$^{+/+}$ and A$_1$AR$^{-/-}$ mouse. White dashed lines, outlines of the tumor; arrows, images (B-D). B, quantification of GI261 glioblastoma volumes in A$_1$AR$^{+/+}$ and A$_1$AR$^{-/-}$ animals revealed significant larger tumor volumes in knockout animals compared with controls 14 days postoperative ($P < 0.01$). C, quantification of microglial/macrophage immunoreactivity showed a higher mean number of immunopositive cells in GI261-bearing A$_1$AR$^{-/-}$ animals compared with GI261-bearing A$_1$AR$^{+/+}$ animals 14 days postoperative ($P < 0.01$). D, Iba-1 immunoreactivity was obviously greater in glioblastoma-bearing A$_1$AR$^{-/-}$ animals compared with glioblastoma-bearing A$_1$AR$^{+/+}$ animals (control) as illustrated by confocal images. Bar, 100 μm.
A1 AR are expressed by glioblastoma cells and up-regulated in tumor-associated microglial cells. To study the cell type–specific expression of A1 AR within glial tumors, we used a specific antibody against A1 AR in combination with cell type–specific markers. Specificity of the antibody for A1 AR was investigated by immunocytochemistry on brain tissue from wild-type and A1 AR−/− mice (see Supplementary Fig. S1). The inoculated tumor cells, either F98 or Gl261 cells, were labeled by stable transfection with enhanced GFP (EGFP) or dsred, brain macrophages/microglial cells with antibody against CD11b, and astrocytes by using a transgenic animal with GFAP promoter-driven EGFP expression. About half of the F98 glioma cells inoculated into Fisher CD344 rats were immunopositive for A1 AR, indicating that tumor cells proper express A1 AR (Fig. 2B and C). Similarly, 60% of the Gl261 cells inoculated into C57BL/6 mice were immunopositive for A1 AR. Close to and within the tumor, almost all (90%) of the CD11b+ cells were immunolabeled for A1 AR (Fig. 2B and C). In contrast, immunoreactivity of A1 AR was low in the contralateral hemisphere (data not shown), indicating that microglia associated with the glioblastoma increase A1 AR expression. As a second approach to study A1 AR expression in microglia, we injected dsred-labeled Gl261 glioblastoma cells into transgenic C57BL/6 mice expressing GFP under the control of the CX3CR1 promoter, thus specifically labeling microglia cells (Fig. 3). Similar as observed in the rat model, microglia in the vicinity of the tumor were immunolabeled for A1 AR expression (Fig. 3B and C). In contrast, immunoreactivity of A1 AR was low on the contralateral site (data not shown).

Astrocytes were not immunolabeled for A1 AR expression close to the tumor zone (Fig. 4A). Because nestin-positive progenitor cells have recently been shown to be attracted by glioblastomas (18), we studied tumors in transgenic animals expressing GFP under the control of the nestin promoter. These nestin-positive progenitor cells were not immunolabeled for A1 AR (Fig. 4B). In conclusion, A1 AR are expressed by microglial cells associated with glioblastoma cells and by glioblastoma cells proper.

Human glioblastoma samples exhibit expression of A1 AR in microglia and tumor cells. In tumor samples from glioblastoma patients (n = 5), A1 AR-positive cells were abundantly detected (Fig. 4C). Isoclin B4 as a marker for microglial cells was colabeled with A1 AR in 90% of the cells from the human glioblastoma probes (Fig. 4C). Only in a few A1 AR-positive cells (~5%) from human glioblastoma probes, we detected coexpression of markers for the oligodendrocyte lineage, such as the glycoprotein O4 (data not shown), for the neuronal lineage marker Neu-N (data not shown), and for the endothelial marker von Willebrand factor (data not shown). GFAP is a classic marker for astrocytes and putative resident stem cells but also labels glioblastoma cells. In the present study, GFAP was found in 15% of A1 AR-positive cells (data not shown). S100β as a marker for mature astrocytes was not coexpressed with A1 AR.

Modulation of A1 AR activity influences glioblastoma growth in organotypical brain slice cultures. To test the functional effect of A1 AR activity on glioblastoma growth, we employed an organotypical slice model where we injected glioma cells (24). In contrast, immunoreactivity of A1 AR was low on the contralateral side (data not shown).
and could stimulate or inhibit adenosine receptors. Brain slices (250 μm thick) were cultured for 4 days and 10^5 GFP-labeled Gl261 tumor cells were injected (suspended in 0.1 μL) into the tissue (Fig. 5A). The tumor size was evaluated by measuring the area occupied by the fluorescently labeled Gl261 cells. Daily addition of 100 μmol/L adenosine to the cultivation medium showed a significant inhibition of tumor size to 44% after 4 days compared with a nontreated control group [mean ± SD, 1.06 ± 0.16 mm^2 for the control (n = 10) and 0.46 ± 0.14 mm^2 in the presence of adenosine (n = 14); Fig. 5F]. The specific A1AR agonist CPA (100 nmol/L) had a similar effect on tumor growth [mean ± SD, 0.72 ± 0.09 mm^2 (n = 57) and 1.11 ± 0.1 mm^2 for the control (n = 46); P < 0.05; Fig. 5C]. In summary, stimulation of adenosine receptors significantly impairs tumor growth.

Inhibition of tumor growth mediated by adenosine receptors depends on the presence of microglia. Endogenous microglia can be selectively depleted from cultured organotypical slices by a 24-hour treatment with clodronate-filled liposomes. The other cell types, neurons, oligodendrocytes, and astrocytes are not affected. As reported previously, activated microglia supported glioblastoma growth [mean ± SD, 1.14 ± 0.23 mm^2 (n = 37); P < 0.05; Fig. 5D]. Similarly, activation of A1AR with the specific agonist CPA (100 nmol/L) in microglia-depleted slices also increased tumor size as studied 4 days after inoculation [mean ± SD, 0.8 ± 0.15 mm^2 (n = 45) for the microglia-depleted control and 1.95 ± 0.15 mm^2 for 100 μmol/L adenosine (n = 45); P < 0.05; Fig. 5D]. In an additional approach, we evaluate modulation of tumor growth in brain slices of A1AR+/− mice comparing with A1AR+/+ mice. As expected, tumor growth in organotypical brain slice cultures of A1AR+/− mice exceeded significantly tumor growth in A1AR+/+ mice [mean ± SD, 1.25 ± 0.16 mm^2 (n = 8) for the A1AR+/− control and 2.12 ± 0.23 mm^2 for the A1AR+/+; P < 0.05; Fig. 6A]. Daily addition of either 100 μmol/L adenosine or 100 nmol/L CPA do not influence tumor growth within brain slice cultures of A1AR+/− mice. We conclude that CPA and adenosine specifically act on A1AR and that the tumor-reducing effect of adenosine requires the presence of microglia.

Microglia increase expression of A1AR in coculture with glioblastoma cells. A1AR expression immunoreactivity was detected in all cultured, purified microglial cells and in cultured Gl261 and F98 glioblastoma cells. After coculturing Gl261 with microglial cells for 4 days, the A1AR immunolabeling in microglial cells was stronger compared with control microglial cells, indicating that the presence of tumor cells up-regulates the expression of A1AR in microglia (data not shown). No changes in the degree and pattern of A1AR expression immunoreactivity were observed after coculturing Gl261 cells with astrocytes and coculturing microglia with astrocytes (data not shown).

Figure 4. Distribution of A1AR expression in human glioblastoma probe and glioblastoma-induced knock-in mice labeled for GFAP and nestin. To examine the possible role of reactive astrocytes and neural precursor cells in the process of glioblastoma development, Gl261-dsred cells induced glioblastomas (red) in the caudate putamen of P25 GFAP-GFP mice and nonlabeled Gl261-induced glioblastomas in the caudate putamen of P25 nestin-GFP (green) were evaluated 14 days postoperatively for A1AR immunoreactivity. Phase-contrast images corresponding to confocal images displaying the brain morphology 14 days after tumor inoculation. White dashed lines, outlines of the tumor; black dashed boxes, confocal images. A, Gl261-dsred-induced glioblastomas (red) are surrounded by reactive astrocytes identified by GFAP-GFP labeling (green). A1AR immunoreactivity does not exhibit a confine colabeling with reactive astrocytes. B, induced nonlabeled Gl261 glioblastomas are densely enwrapped by neural progenitors cells identified by nestin-GFP labeling. A1AR immunoreactivity does not exhibit a confine colabeling with neural precursor cells. Bar, 25 μm (A and B). C, specimen of a neuropathologically confirmed glioblastomas was obtained during planned neurosurgical excisions. There is an abundant labeling for A1AR in the tumor probe. Colabeling with isoclin B4 as a marker for microglia reveals a strict colocalization with A1AR immunoreactivity.

Activation of the A1AR prominently decreased the glioma-stimulated activity of MMP-2 in microglia. In supernatants of microglia cell cultures, being either stimulated or nonstimulated with glioma conditioned medium, we measured the activity of MMP. Gelatin-degrading MMPs are the main mediators of matrix-degrading activity in gliomas and substantially contribute to glioma cell invasion. In gelatin zymographies, we observed that microglia abundantly release active MMP-2 after stimulation with glioma conditioned medium (Fig. 6B). The glioma-stimulated increase in MMP-2 activity was blunted by costimulation with 100 μmol/L adenosine.

Discussion

Adenosine-impaired tumor growth is mediated by microglia. Our present data indicate that the deletion of functional adenosine receptors, specifically A1AR, results in an increase in brain tumor...
growth. This implies that adenosine acting via A1AR impairs glioma growth. In the context of glioma, A1ARs are prominently expressed by the tumor cells and those microglial cells associated with the glioma cells. In our experimental approach using the A1AR-deficient mouse as a tumor host, we highlight the importance of the microglial cells for mediating the A1AR effect. This does not exclude that adenosine can also affect the glioma cells directly. The microglial cells were accumulating at the tumor site and this accumulation was even more pronounced in the A1AR-deficient mice. The importance of microglial A1AR is further supported by our brain slice model with tumor impairment was only observed in the presence of microglial cells. We thereby confirmed the observation that the presence of microglial cells per se is tumor promoting (24). There was no significant change in the population of astrocytes or neural progenitor cells. The latter is of particular interest because we recently showed that neural progenitor cells are attracted to tumors or to gliomas and attenuate tumor growth (18).

Microglia and glioma cells express adenosine receptors. The presence of adenosine receptors has been reported previously on astrocytoma cells (21, 23) using an A1AR-specific ligand. A recent positron emission tomography study indicates that specific binding sites for A1AR ligands are associated with experimental glioblastoma (27). The presence of adenosine receptors on microglia is well established and some functional implications of their activation have become apparent. Cultured rat microglial cells express A2ARs, because its specific agonist CGS 21680 triggered the expression of K+ channels that are linked to microglial activation (28). In contrast, A1AR stimulation in rat microglia triggers the expression of nerve growth factor and its release, thereby exerting a neuroprotective effect (29). Moreover, cyclooxygenase-2 expression of nerve growth factor and its release, thereby exerting a neuroprotective effect (29). Moreover, cyclooxygenase-2 expression of nerve growth factor and its release, thereby exerting a neuroprotective effect (29).

The potential source for extracellular adenosine is most likely ATP, which is released activity dependent from presynaptic and postsynaptic terminals of neurons and also from glial cells (32). In the extracellular space, adenosine is generated from ATP after dephosphorylation by specific ectoenzymes (e.g., CD39 and CD73). These ectoenzymes represent a highly organized enzymatic cascade for the regulation of nucleotide-mediated signaling. They control the rate of nucleotide (e.g., ATP) degradation and nucleoside (e.g. adenosine) formation (33). Microglial cells express specific ectonucleotidase isoforms, CD39 and CD73, which are not expressed by any other cell type in the brain. Due to this specific expression, both molecules have served as microglia-specific markers even long before their functional importance has been recognized (34–36).
A1AR deficiency leads to an increased microglial density at gliomas. Our data indicate that loss of A1AR leads to an increase of tumor-associated microglia, which can be due to infiltration and/or proliferation. The role of adenosine for microglial proliferation remains controversial. One study reports that adenosine stimulates the proliferation of microglial cells through a mechanism that involves the simultaneous stimulation of A1AR and A2AR (37). By contrast, adenosine has been reported to inhibit proliferation of microglial cells: phorbol 12-myristate 13-acetate–stimulated microglial proliferation is reduced following treatment with an A1AR antagonist (38). Moreover, adenosine receptor stimulation by the A1AR can also cause microglial apoptosis (39).

Adenosine levels in the extracellular fluid are lower in human glioma tissue compared with control tissue (i.e., 1.5 and 3 μmol/L, respectively). These values were obtained from human gliomas of high-grade malignancy and measured by brain microdialysis coupled to high-performance liquid chromatography (25). Whether this rather small difference causes the accumulation of microglia close to tumors is speculative.

A1AR mediates tissue protection. Recent studies support the idea that adenosine receptors: specifically, the A1AR are good targets for drug development in several diseases that affect the CNS (40). A1AR deficiency aggravates experimental allergic encephalomyelitis (4) and it has been repeatedly shown that adenosine can protect tissues against the negative consequences of hypoxia or ischemia (41) mainly by acting on A1AR. Hence, survival after a hypoxic challenge may be reduced if A1ARs are absent or blocked (17).

The tissue protective effect of A1AR has been implicated in experimental paradigms using the A1AR-deficient mice. In a model of renal ischemia and reperfusion injury, A1AR-deficient mice exhibited an increased production of proinflammatory cytokines and also augmented axonal injury (4). Both studies concluded that A1AR serves anti-inflammatory functions that regulate subsequent tissue damage. Furthermore, MMP-9 and MMP-12 are significantly elevated in A1AR-deficient mice (4). Indeed, MMPs play an important role in glial progression; as we showed recently, expression of MMPs by microglia has an effect on tumor growth (24).

Adenosine-regulated glioma invasion is due to the activity of extracellular proteases. Matrix degradation by MMPs is an important prerequisite for glioblastoma invasion (42). It was shown previously that A1AR activation on microglia/macrophages inhibits the production of not only cytokines, such as interleukin-1β, but also MMPs, such as MMP 12 (4). In our present study, we observed that activation of the A1AR prominently decreased the glioma-stimulated activity of MMP-2 in microglia. Above, we have described that A1AR-deficient microglia exhibits increased chemotraction toward gliomas and that the enhanced accumulation of microglia is associated with increased tumor size. The zymographies suggest that A1AR blockade on microglia may facilitate their attraction toward the tumor because these cells have higher matrix-degrading activity and may therefore faster arrive at the lesion. However, this increased matrix degradation by microglia may also create an environment, in which glioma cells can easier invade into the surrounding parenchyma and thereby facilitates tumor growth.

Results from this study show that the A1AR plays an antitumorigenic role mediated by microglia cells in the development of glioblastomas. If we better understand how the pathways of A1AR signaling modulate glioblastoma development, it may ultimately lead to concepts to reduce the progression of this disease.

Acknowledgments

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