DOI:10.1158/0008-5472.CAN-06-0365

Research Article

A_1 Adenosine Receptors in Microglia Control Glioblastoma-Host Interaction

Michael Synowitz,[1,2] Rainer Glass,[1] Katrin Färber,[1] Darko Markovic,[1] Golo Kronenberg,[3] Ken Herrmann,[1] Juergen Schnermann,[4] Christiane Nolte,[1] Nico van Rooijen,[5] Juergen Kiwit,[2] and Helmut Kettenmann[1]

[1]Cellular Neuroscience Group, Max Delbrück Center for Molecular Medicine; [2]Department of Neurosurgery, Helios Hospital Berlin; [3]Department of Psychiatry, Free University, Berlin, Germany; [4]National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland; and [5]Department of Molecular Cell Biology, Faculty of Medicine, Amsterdam, the Netherlands

Abstract

We report that experimental glioblastoma grow more vigorously in A_1 adenosine receptor (A_1AR)–deficient mice associated with a strong accumulation of microglial cells at and around the tumors. A_1ARs were prominently expressed in microglia associated with tumor cells as revealed with immunocytochemistry but low in microglia in the unaffected brain tissue. The A_1AR 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. A_1AR 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 A_1AR 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 (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR) each with varying ligand affinities, tissue distributions, and signal transduction mechanisms. The A_1AR has a high affinity for adenosine and has been implicated in both proinflammatory and anti-inflammatory aspects of disease processes. On one side, A_1AR signaling can promote neutrophil (1) and monocyte (2, 3) activation, whereas, on the other side, A_1AR 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 A_1AR activation by high levels of adenosine in the lung, a surprising and important finding in light of the fact that A_1AR antagonists are being investigated as a potential treatment for asthma (11).

In the central nervous system (CNS), the A_1AR is highly expressed on microglia/macrophages and neurons (12). In the latter, A_1AR is coupled to activation of K^+ channels (13) and inhibition of Ca^{2+} channels (14), both mechanisms that attenuate neuronal excitability. By reducing excitotoxicity, adenosine can act as a neuroprotective factor. Because A_1 adenosine receptors (A_1AR) are expressed throughout the brain (15), adenosine has the potential to be involved in different brain pathologies. Although A_1ARs modulate physiologic functions, A_1AR-deficient mice show no obvious abnormal behavior (16, 17). However, exposed to pathophysiologic conditions, such as hypoxia, A_1AR-deficient mice show more neuronal damage and have a lower survival rate. It was therefore concluded that A_1ARs are primarily important in mediating effects of adenosine during pathophysiologic conditions (16, 17). In the present study, we have addressed the question whether A_1ARs 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 A_1AR-null mice ($A_1AR^{-/-}$) and littermate wild-type ($A_1AR^{+/+}$) controls [both with SV129/C57BL/6 background; bred as described previously by Sun et al. (22)]. Briefly, heterozygous mice were bred to obtain homozygous A_1AR-deficient mice and wild-type littermates. Animals were genotyped after performing PCR on samples from tail cuts. Message for the A_1AR was amplified by the following primers: A_1AR sense 5′-GTACATCTCGGCCTTCCAGG-3′ and antisense 5′-GAGAATACCTGGCTGACTAG-3′. Message for a neomycin resistance cassette replacing the A_1AR was amplified by NeoR sense 5′-ACAACAGACAATCGGCTGCTCTGATG-3′ and antisense 5′-TGCGCGCCTTGAGCCTGGCGAAC-3′.

Glioblastoma cell lines. Gl261 (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 DMEM (Invitrogen, Heidelberg, Germany) with additives.

Microglial cell cultures. Microglial cultures were prepared from cerebral cortex of newborn C57BL/6 mice as described previously (23).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

M. Synowitz, R. Glass, and K. Färber contributed equally to this work.

Requests for reprints: Helmut Kettenmann, Max Delbrück Center for Molecular Medicine Berlin-Buch (MDC) Robert Rössle Strasse 10, 13092 Berlin, Germany. Phone: 49-30-9406-3325; Fax: 49-30-9406-3819; E-mail: kettenmann@mdc-berlin.de.

©2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-0365

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

In brief, the forebrain 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 10^6/well. Cultures usually contained >95% microglial cells, which could be stained with *Griffonia simplicifolia* isolectin 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 A_1AR. 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_1AR (Chemicon, Temecula, CA); guinea pig anti-GFAP (Advanced ImmunoChemical, Long Beach, CA); rabbit anti-GFP (Abcam, Cambridgeshire, United Kingdom); goat anti-GFP (DPC, Bad Nauheim, Germany); mouse anti-dsred (Abcam); rabbit anti-S100β (Swant, 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 diazabicyclo-octane 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 colocalization images were processed with Volocity version 2.6.1 (Volocity, 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 3502, 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 (medium 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 mol/L Tris in DMEM. Adenosine and N^6-cyclopentyladenosine (CPA) were added daily.

Inoculation of Gl261 and F98 cells into rats and mice. Anesthetized CD344 Fisher rats, nestin-GFP, GFAP-GFP, CX3CR1-GFP, A_1AR wild-type, A_1AR 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 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 (10^4 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 10^4 GFP-transfected Gl261 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 GOT 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 diazabicyclo-octane 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 $CaCl_2$, 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.

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

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_1AR 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 in $A_1AR^{-/-}$ mice and $A_1AR^{+/+}$ littermate controls. With this approach, we deleted the A_1AR 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 $A_1AR^{-/-}$ mice was significantly larger compared with $A_1AR^{+/+}$ mice [mean ± SD, 2.96 ± 0.48 mm^3 for control (n = 14) and 5.21 ± 0.53 mm^3 for $A_1AR^{-/-}$ mice (n = 12); $P < 0.01$; Fig. 1*B*]. 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 $A_1AR^{-/-}$ and $A_1AR^{+/+}$ mice. Immunoreactivity for the macrophage/microglia marker Iba-1 revealed an accumulation of Iba-1-positive cells at the tumor border (Fig. 1*C* and *D*). In $A_1AR^{-/-}$ 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 $A_1AR^{+/+}$ (n = 15) and 547 ± 123 cells/mm^2 for $A_1AR^{-/-}$ mice (n = 15); $P < 0.01$; Fig. 1*C*]. No differences in the GFAP-positive cell population was observed comparing $A_1AR^{-/-}$ and $A_1AR^{+/+}$ (data not shown). These results imply that A_1AR modulate tumor growth and that microglial cells are the cellular candidates to mediate this effect.

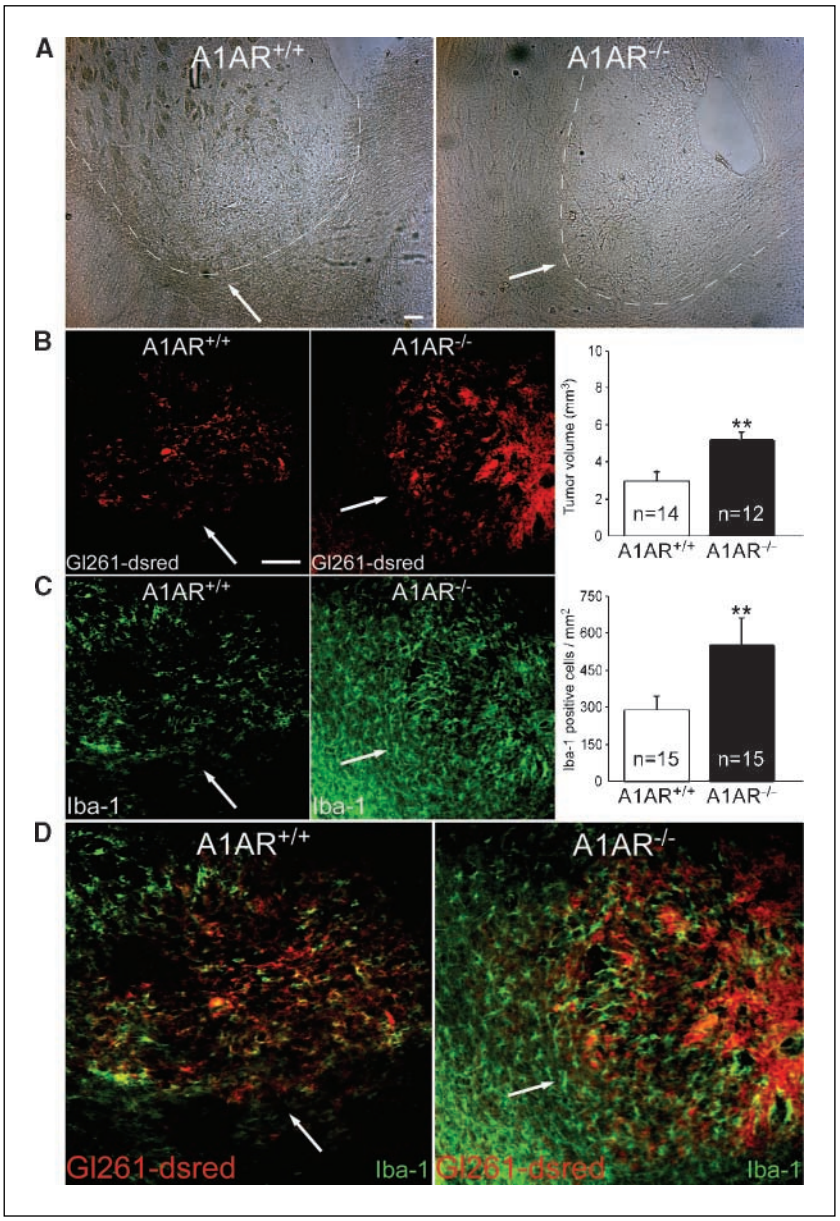

Figure 1. Gl261 glioblastoma growth in $A_1AR^{+/+}$ and $A_1AR^{-/-}$ animals. *A,* phase-contrast images corresponding to confocal images in (*B-D*) displaying the brain morphology 14 days after tumor inoculation into a $A_1AR^{+/+}$ and $A_1AR^{-/-}$ mouse. *White dashed lines,* outlines of the tumor; *arrows,* images (*B-D*). *B,* quantification of Gl261 glioblastoma volumes in $A_1AR^{+/+}$ and $A_1AR^{-/-}$ 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 Gl261-bearing $A_1AR^{-/-}$ animals compared with Gl261-bearing $A_1AR^{+/+}$ animals 14 days postoperative ($P < 0.01$). *D,* Iba-1 immunoreactivity was obviously greater in glioblastoma-bearing $A_1AR^{-/-}$ animals compared with glioblastoma-bearing $A_1AR^{+/+}$ animals (control) as illustrated by confocal images. Bar, 100 μm.

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

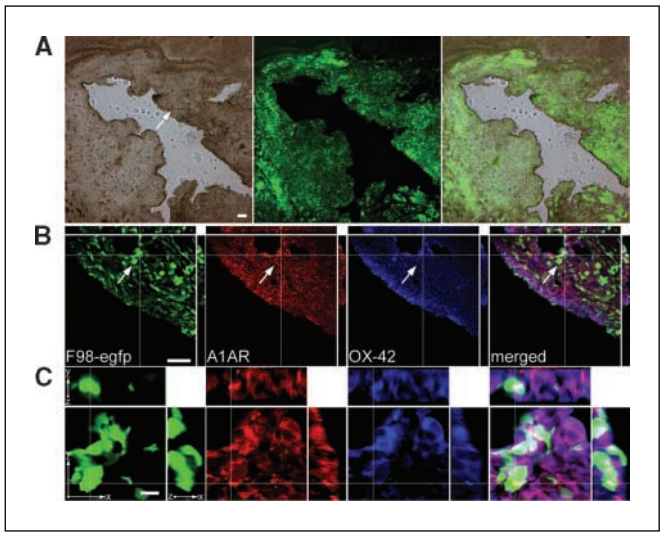

Figure 2. Glioblastoma cells and microglia are the main source of A_1AR expression within glioblastoma host interaction. F98-GFP cells induced glioblastomas (*green*) in the caudate putamen of P50 Fisher CD344 rats, which were examined for cell type–specific markers (*red and blue*) 14 days postoperatively. Colocalization of these markers is shown in the micrographs (*arrows*) and by three-dimensional reconstructions of a single cells. *A,* light micrograph of coronal sections through rat brains bearing GFP-expressing glioblastoma give representative example of tumor size and location. *B,* three-dimensional reconstruction of a *Z* series through the (*A*) labeled region (*arrow*) along the *Z-X* axis (*right narrow*) and *Z-Y* axis (*top narrow*), which confirm that both F98-GFP (*green*) and microglia/macrophages (identified by CD11b labeling; *blue*) indeed colocalize with A_1AR in this cell, which appears yellowish in F98-GFP cells and lilac in CD11b-labeled microglia. Note that almost every CD11b-labeled cells is strong colocalized by A_1AR and the three-dimensional overview confirms that CD11B/A_1AR and GFP/A_1AR are indeed in the same cell. *C,* three-dimensional reconstruction of 40-μm image stack of F98-GFP cells (*green*) and CD11b-labeled microglia/macrophages (*blue*), which both coexpress A_1AR (*red*).

A_1AR are expressed by glioblastoma cells and up-regulated in tumor-associated microglial cells. To study the cell type–specific expression of A_1AR within glial tumors, we used an specific antibody against A_1AR in combination with cell type–specific markers. Specificity of the antibody for A_1AR was investigated by immunocytochemistry on brain tissue from wild-type and $A_1AR^{-/-}$ 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 A_1AR, indicating that tumor cells proper express A_1AR (Fig. 2*B* and *C*). Similarly, 60% of the Gl261 cells inoculated into C57BL/6 mice were immunopositive for A_1AR. Close to and within the tumor, almost all (90%) of the $CD11b^+$ cells were immunolabeled for A_1AR (Fig. 2*B* and *C*). In contrast, immunoreactivity of A_1AR was low in the contralateral hemisphere (data not shown), indicating that microglia associated with the glioblastoma cells increase A_1AR expression. As a second approach to study A_1AR 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*A*). Similar as observed in the rat model, microglia cells in the vicinity of the tumor were immunolabeled for A_1AR expression (Fig. 3*B* and *C*). In contrast, immunoreactivity of A_1AR was low on the contralateral site (data not shown).

Astrocytes were not immunolabeled for A_1AR expression close to the tumor zone (Fig. 4*A*). 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 A_1AR (Fig. 4*B*). In conclusion, A_1AR are expressed by microglial cells associated with glioblastoma cells and by glioblastoma cells proper.

Human glioblastoma samples exhibit expression of A_1AR in microglia and tumor cells. In tumor samples from glioblastoma patients (n = 5), A_1AR-positive cells were abundantly detected (Fig. 4*C*). Isolectin B4 as a marker for microglial cells was colabeled with A_1AR in 90% of the cells from the human glioblastoma probes (Fig. 4*C*). Only in a few A_1AR-positive cells (<5%) from human glioblastoma probes, we detected coexpression of markers for the oligodendrocyte lineage, such as the glycopeptide 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 A_1AR-positive cells (data not shown). S100β as a marker for mature astrocytes was not coexpressed with A_1AR.

Modulation of A_1AR activity influences glioblastoma growth in organotypical brain slice cultures. To test the functional effect of A_1AR activity on glioblastoma growth, we employed an organotypical slice model where we injected glioma cells (24)

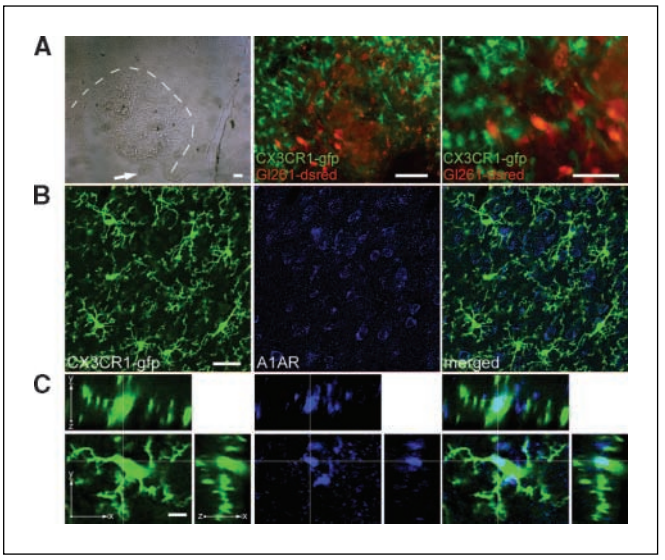

Figure 3. Verification of A_1AR expression on microglia cells in CX3CR1-GFP-labeled microglia knock-in mice. Gl261-dsred cells induced glioblastomas (*red*) in the caudate putamen of P25 CX3CR1-GFP mice (microglia-labeled CX3CR1-GFP cells; *green*), which express A_1AR cell type–specific marker (*blue*) 14 days postoperatively. Colocalization with CX3CR1-GFP cells (*microglia*) is shown by three-dimensional reconstructions of single cells. *A,* phase-contrast image displaying the brain morphology 14 days after tumor inoculation into CX3CR1-GFP mouse. *White dashed lines,* outlines of the tumor; *arrows,* confocal images. Fluorescence microscope images in different magnification illustrate the strong association of CX3CR1-GFP-labeled microglia to the Gl261-dsred-induced glioblastoma especially in the tumor margin. *B,* labeling A_1AR expression revealed a clear colocalization of CX3CR1-GFP-labeled microglia (*green*) and A_1AR immunoactivity (*blue*). *C,* three-dimensional reconstruction of a *Z* series through CX3CR1-GFP-positive cell along the *Z-X* axis (*right narrow*) and *Z-Y* axis (*top narrow*), which confirms that A_1AR (*blue*) and GFP (*green*) are indeed present in the same cell (*light blue*). Bar, 75 μm (*A*), 25 μm (*B*), and 7 μm (*C*).

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

and could stimulate or inhibit adenosine receptors. Brain slices (250 μm thick) were cultured for 4 days and 10^4 GFP-labeled Gl261 tumor cells were injected (suspended in 0.1 μL) into the tissue (Fig. 5*A*). 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. 5*B*]. The specific A_1AR 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. 5*C*]. 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 glial tumor growth resulting in significant smaller tumors in microglia-depleted slices compared with control slices (24). This served us as an internal control. Tumor cells were injected 3 days after liposome treatment and the size of the tumor bulk was evaluated in both slices with and devoid of microglia. We compared control slices with those receiving a daily treatment with adenosine and the specific A_1AR agonist CPA. As expected, depletion of endogenous microglial cells in organotypical brain slice cultures leads to a reduction in tumor growth [mean ± SD, 1.25 ± 0.23 mm^2 (n = 61) for the control with microglia and 0.80 ± 0.15 mm^2 for the control devoid of endogenous microglia; $P < 0.05$; Fig. 5*D*]. Activation of adenosine receptors in brain slice cultures devoid of endogenous microglia resulted in a larger tumor size [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 = 37); $P < 0.05$; Fig. 5*D*]. Similarly, activation of A_1AR 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 1.7 ± 0.23 mm^2 (n = 39) for 100 nmol/L CPA; $P < 0.05$; Fig. 5*D*]. In an additional approach, we evaluate modulation of tumor growth in brain slices of $A_1AR^{-/-}$ mice comparing with $A_1AR^{+/+}$ mice. As expected, tumor growth in organotypical brain slice cultures of $A_1AR^{-/-}$ mice exceeded significantly tumor growth in $A_1AR^{+/+}$ mice [mean ± SD, 1.25 ± 0.16 mm^2 (n = 8) for the $A_1AR^{+/+}$ control and 2.12 ± 0.23 mm^2 for the $A_1AR^{-/-}$; $P < 0.05$; Fig. 6*A*]. Daily addition of either 100 μmol/L adenosine or 100 nmol/L CPA do not influence tumor growth within brain slice cultures of $A_1AR^{-/-}$ mice. We conclude that that CPA and adenosine specifically act on A_1AR and that the tumor-reducing effect of adenosine requires the presence of microglia.

Microglia increase expression of A_1AR in coculture with glioblastoma cells. A_1AR 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 A_1AR immunolabeling in microglial cells was stronger compared with control microglial cells, indicating that the presence of tumor cells up-regulates the expression of A_1AR in microglia (data not shown). No changes in the degree and pattern of A_1AR expression immunoreactivity were observed after coculturing Gl261 cells with astrocytes and coculturing microglia with astrocytes (data not shown).

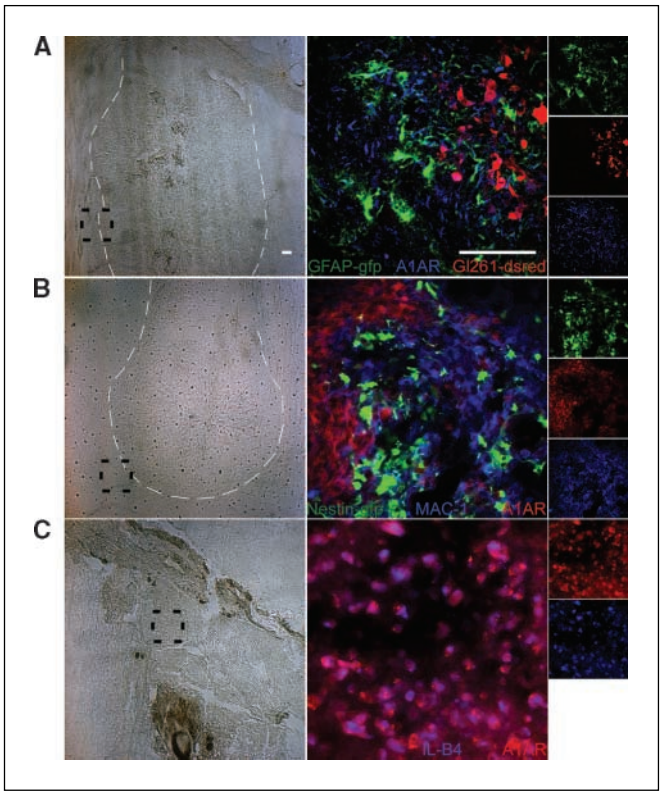

Figure 4. Distribution of A_1AR expression in human glioblastoma probe and glioblastoma-induced knock-in mice labeled for GFAP and nestin. To examine the possible role of reactive astrocytes and neural precursor cells in the process of glioblastoma development, Gl261-dsred cells induced glioblastomas (*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 A_1AR immunoactivity. 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*). A_1AR immunoactivity 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. A_1AR immunoactivity 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 A_1AR in the tumor probe. Colabeling with isolectin B4 as a marker for microglia cells revealed a strict colocalization with A_1AR immunoreactivity.

Activation of the A_1AR 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. 6*B*). 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 A_1AR, results in an increase in brain tumor

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

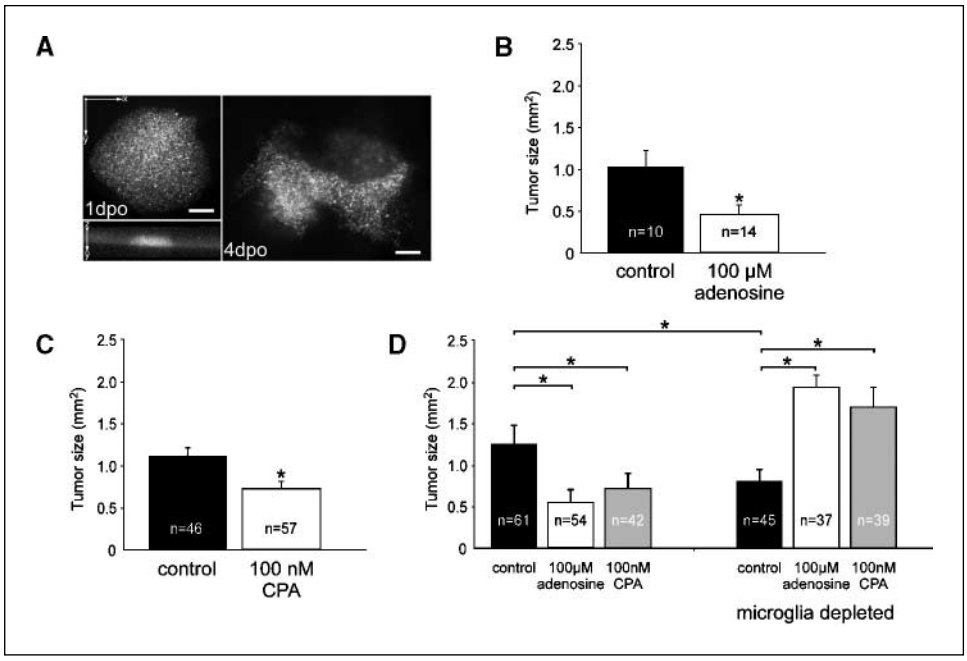

Figure 5. A_1AR modulation influences glial tumor growth observed in organotypical brain slice cultures. Modulation of Gl261-GFP-induced glial tumor growth was evaluated in organotypical brain slice cultures after 4 days. *A,* fluorescence microscope images illustrate a compact Gl261-GFP tumor in all three planes 1 day after inoculation. Tumor size after 4 days depicts the measurability of tumor bulk size. *B,* daily treatment of slice cultures with 100 μmol/L adenosine leads to a significant tumor inhibition after 4 days. *C,* daily treatment of Gl261-GFP-bearing brain slice cultures with specific A_1AR agonist CPA revealed significant influence on glial tumor growth, indicating a functional role of A_1AR expression in the process of glioblastoma-host interaction. Bar, 100 μm (*A, left*) and 250 μm (*A, right*). To evaluate the possible role of microglial cells in the A_1AR-mediated influence on glial tumor growth, we depleted selectively microglia in organotypical brain slice cultures as described. *D,* depletion of microglia in Gl261-bearing brain slice cultures reversed significantly the observed effect of A_1AR stimulation in microglia-containing slices ($P < 0.05$).

growth. This implies that adenosine acting via A_1AR impairs glioma growth. In the context of glioma, A_1ARs are prominently expressed by the tumor cells and those microglial cells associated with the glioma cells. In our experimental approach using the A_1AR-deficient mouse as a tumor host, we highlight the importance of the microglial cells for mediating the A_1AR 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 A_1AR-deficient mice. The importance of microglial A_1AR is further supported by our brain slice model where 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 A_1AR-specific ligand. A recent positron emission tomography study indicates that specific binding sites for A_1AR 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 A_2ARs, because its specific agonist CGS 21680 triggered the expression of K^+ channels that are linked to microglial activation (28). In contrast, A_2AR stimulation in rat microglia triggers the expression of nerve growth factor and its release, thereby exerting a neuroprotective effect (29). Moreover, cyclooxygenase-2 expression in rat microglia is induced by A_2ARs resulting in the release of prostaglandin (30). Hammarberg et al. (31) provided evidence for functional A_3ARs in mouse microglia cells, whereas A_1ARs were not detected in this study. Our results based on our immunocytochemical data, however, indicate that microglial cells express A_1AR.

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).

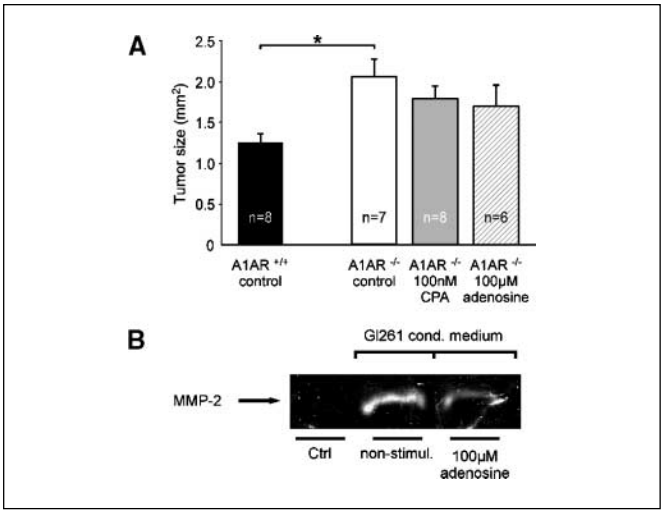

Figure 6. *A,* tumor growth in organotypical brain slice cultures of $A_1AR^{-/-}$ mice exceeded significantly tumor growth in $A_1AR^{+/+}$ mice. Daily addition of either 100 μmol/L adenosine or 100 nmol/L CPA does not influence tumor growth within brain slice cultures of $A_1AR^{-/-}$ mice. *B,* gelatin zymography for MMP-2 activity was analyzed from supernatant obtained from microglial cultures (*left*), from microglial cultures stimulated with Gl261-conditioned medium (*middle*), and from microglia cultures mixed with supernatant from Gl261 cultures and stimulated by 100 μmol/L adenosine (*right*).

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

A_1AR deficiency leads to an increased microglial density at gliomas. Our data indicate that loss of A_1AR 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 A_1AR and A_2AR (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 A_1AR agonist (38). Moreover, adenosine receptor stimulation by the A_1AR 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.

A_1AR mediates tissue protection. Recent studies support the idea that adenosine receptors; specifically, the A_1AR are good targets for drug development in several diseases that affect the CNS (40). A_1AR 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 A_1AR. Hence, survival after a hypoxic challenge may be reduced if A_1ARs are absent or blocked (17).

The tissue protective effect of A_1AR has been implicated in experimental paradigms using the A_1AR-deficient mice. In a model of renal ischemia and reperfusion injury, A_1AR-deficient mice exhibited an increase production of proinflammatory mediators and showed an increased renal injury (6, 7). Similarly, in a model of experimental allergic encephalomyelitis, A_1AR deficiency led to an increased neuroinflammation and demyelination and also augmented axonal injury (4). Both studies concluded that A_1AR serves anti-inflammatory functions that regulate subsequent tissue damage. Furthermore, MMP-9 and MMP-12 are significantly elevated in A_1AR-deficient mice (4). Indeed, MMPs play an important role in glioma 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 A_1AR 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 A_1AR prominently decreased the glioma-stimulated activity of MMP-2 in microglia. Above, we have described that A_1AR-deficient microglia exhibits increased chemoattraction toward gliomas and that the enhanced accumulation of microglia is associated with increased tumor size. The zymographies suggest that A_1AR 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 A_1AR plays an antitumorigenic role mediated by microglia cells in the development of glioblastomas. If we better understand how the pathways of A_1AR signaling modulate glioblastoma development, it may ultimately lead to concepts to reduce the progression of this disease.

Acknowledgments

Received 1/30/2006; revised 6/15/2006; accepted 7/6/2006.

Grant support: Bundesministerium für Bildung und Forschung (Förderkennzeichen) 01GZ0304 (H. Kettenmann) and Stiftung Neurochirurgische Forschung der Deutschen Gesellschaft für Neurochirurgie (M. Synowitz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Salmon JE, Cronstein BN. Fcγ receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy. A_1 receptors are stimulatory and A_2 receptors are inhibitory. J Immunol 1990;145:2235–40.
2. Merrill JT, Shen C, Schreibman D, et al. Adenosine A_1 receptor promotion of multinucleated giant cell formation by human monocytes: a mechanism for methotrexate-induced nodulosis in rheumatoid arthritis. Arthritis Rheum 1997;40:1308–15.
3. Salmon JE, Brogle N, Brownlie C, et al. Human mononuclear phagocytes express adenosine A_1 receptors. A novel mechanism for differential regulation of Fcγ receptor function. J Immunol 1993;151:2775–85.
4. Tsutsui S, Schnermann J, Noorbakhsh F, et al. A_1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. J Neurosci 2004;24:1521–9.
5. Liao Y, Takashima S, Asano Y, et al. Activation of adenosine A_1 receptor attenuates cardiac hypertrophy and prevents heart failure in murine left ventricular pressure-overload model. Circ Res 2003;93:759–66.
6. Lee HT, Xu H, Nasr SH, Schnermann J, Emala CW. A_1 adenosine receptor knockout mice exhibit increased renal injury following ischemia and reperfusion. Am J Physiol Renal Physiol 2004;286:F298–306.
7. Lee HT, Gallos G, Nasr SH, Emala CW. A_1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. J Am Soc Nephrol 2004;15:102–11.
8. Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM, Vizi ES. Adenosine receptor agonists differentially regulate IL-10, TNF-α, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. J Immunol 1996;157:4634–40.
9. Le MO, Stordeur P, Schandene L, et al. Adenosine enhances IL-10 secretion by human monocytes. J Immunol 1996;156:4408–14.
10. Sajjadi FG, Takabayashi K, Foster AC, Domingo RC, Firestein GS. Inhibition of TNF-α expression by adenosine: role of A_3 adenosine receptors. J Immunol 1996;156:3435–42.
11. Sun CX, Young HW, Molina JG, Volmer JB, Schnermann J, Blackburn MR. A protective role for the A_1 adenosine receptor in adenosine-dependent pulmonary injury. J Clin Invest 2005;115:35–43.
12. Johnston JB, Silva C, Gonzalez G, et al. Diminished adenosine A_1 receptor expression on macrophages in brain and blood of patients with multiple sclerosis. Ann Neurol 2001;49:650–8.
13. Trussell LO, Jackson MB. Adenosine-activated potassium conductance in cultured striatal neurons. Proc Natl Acad Sci U S A 1985;82:4857–61.
14. MacDonald RL, Skerritt JH, Werz MA. Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. J Physiol 1986;370:75–90.
15. Dunwiddie TV. The physiological role of adenosine in the central nervous system. Int Rev Neurobiol 1985;27:63–139.
16. Gimenez-Llort L, Fernandez-Teruel A, Escorihuela RM, et al. Mice lacking the adenosine A_1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci 2002;16:547–50.
17. Johansson B, Halldner L, Dunwiddie TV, et al. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A_1 receptor. Proc Natl Acad Sci U S A 2001;98:9407–12.
18. Glass R, Synowitz M, Kronenberg G, et al. Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. J Neurosci 2005;25:2637–46.
19. Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 2003;130:391–9.
20. Nolte C, Matyash M, Pivneva T, et al. GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue. Glia 2001;33:72–86.
21. Jung S, Aliberti J, Graemmel P, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research

DOI:10.1158/0008-5472.CAN-06-0365

deletion and green fluorescent protein reporter gene insertion. Mol Cell Biol 2000;20:4106–14.
22. Sun D, Samuelson LC, Yang T, et al. Mediation of tubuloglomerular feedback by adenosine: evidence from mice lacking adenosine 1 receptors. Proc Natl Acad Sci U S A 2001;98:9983–8.
23. Prinz M, Hanisch UK. Murine microglial cells produce and respond to interleukin-18. J Neurochem 1999;72:2215–8.
24. Markovic DS, Glass R, Synowitz M, Rooijen N, Kettenmann H. Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. J Neuropathol Exp Neurol 2005; 64:754–62.
25. van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994;174:83–93.
26. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 1980;102:196–202.
27. Bauer A, Langen KJ, Bidmon H, et al. ^{18}F-CPFPX PET identifies changes in cerebral A_1 adenosine receptor density caused by glioma invasion. J Nucl Med 2005;46: 450–4.
28. Kust BM, Biber K, van CD, Gebicke-Haerter PJ. Regulation of K^+ channel mRNA expression by stimulation of adenosine A_2A-receptors in cultured rat microglia. Glia 1999;25:120–30.
29. Heese K, Fiebich BL, Bauer J, Otten U. Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A_2A-receptors. Neurosci Lett 1997; 231:83–6.
30. Fiebich BL, Biber K, Lieb K, et al. Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_2A-receptors. Glia 1996;18:152–60.
31. Hammarberg C, Schulte G, Fredholm BB. Evidence for functional adenosine A_3 receptors in microglia cells. J Neurochem 2003;86:1051–4.
32. Fields RD, Burnstock G. Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci 2006;7:423–36.
33. Plesner L. Ecto-ATPases: identities and functions. Int Rev Cytol 1995;158:141–214.
34. Braun N, Sevigny J, Robson SC, et al. Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/ CD39 expression to microglia and vasculature of the brain. Eur J Neurosci 2000;12:4357–66.
35. Schnitzer J. Enzyme-histochemical demonstration of microglial cells in the adult and postnatal rabbit retina. J Comp Neurol 1989;282:249–63.
36. Schoen SW, Graeber MB, Kreutzberg GW. 5′-Nucleotidase immunoreactivity of perineuronal microglia responding to rat facial nerve axotomy. Glia 1992;6:314–7.
37. Gebicke-Haerter PJ, Christoffel F, Timmer J, Northoff H, Berger M, van Calker D. Both adenosine A_1- and A_2-receptors are required to stimulate microglial proliferation. Neurochem Int 1996;29:37–42.
38. Si QS, Nakamura Y, Schubert P, Rudolphi K, Kataoka K. Adenosine and propentofylline inhibit the proliferation of cultured microglial cells. Exp Neurol 1996;137:345–9.
39. Ogata T, Schubert P. Programmed cell death in rat microglia is controlled by extracellular adenosine. Neurosci Lett 1996;218:91–4.
40. Fredholm BB, Chen JF, Masino SA, Vaugeois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu Rev Pharmacol Toxicol 2005;45:385–412.
41. Fredholm BB. Adenosine and neuroprotection. Int Rev Neurobiol 1997;40:259–80.
42. Rao JS. Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 2003;3:489–501.

Downloaded from cancerres.aacrjournals.org on April 10, 2012
Copyright © 2006 American Association for Cancer Research