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Cannabinoid receptors in human astroglial tumors

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Abstract

In animal models, cannabinoids are reported to inhibit the growth of tumors, including gliomas. These effects have been claimed to be mediated via cannabinoid receptors 1 and 2 (CB1, CB2). To elucidate a possible relevance for treatment of human gliomas, we investigated receptor subtype expression in surgical material of solid human astrocytomas, gliomas and cultivated glioma cells by quantitative reverse transcriptase polymerase chain reaction, western blot and immunohistochemistry and assayed their functionality. In normal brain, cultivated glioma cells and solid tumors, CB1 mRNA was expressed to a much greater extent than CB2, which in some samples was even undetectable. Expression of both receptor subtypes was unrelated to malignancy, varied between patients, and was not significantly increased in relation to normal brain tissues. In normal brain, CB1 protein was localized on astroglial and other cell types; in gliomas, it was found on astroglial/glioma cells. CB2 protein was detected on microglial cells/macrophages but rarely on astroglial cells. Functionally, CB1 receptor agonists reduced elevated cyclic AMP levels and slightly reduced proliferation of glioma cells *in vitro*, but did not induce apoptosis. We conclude that cannabinoid therapy of human gliomas targets not only receptors on tumor, but also on other cell types. Therefore, complex and potential side-effects should be considered carefully.

Keywords: astrocytomas, cannabinoid receptors, gliomas, proliferation, real-time reverse transcriptase polymerase chain reaction, signal transduction.

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Cannabinoids, the active components of *Cannabis sativa* and their derivatives, have a wide spectrum of pharmacological effects exerted through two specific plasma membrane G-protein-coupled receptors, cannabinoid receptors 1 and 2 (CB1 and CB2). Their stimulation induces a variety of intracellular signal-transducing effects including the inhibition of adenylate cyclase, influence on ion channels, stimulation of extracellular-signal-regulated kinase, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and apotosis-related (ceramide) pathways (Howlett *et al*. 2002).

Several recent reports state that cannabinoids inhibit the growth of various types of cancer cells *in vitro* and *in vivo* (Guzman 2003). In the case of gliomas, *in vitro* and animal experimental studies have shown that cannabinoids can induce apoptosis and reduce glioma growth (Sanchez *et al*. 1998, 2001; Galve-Roperh *et al*. 2000; Blazquez *et al*. 2003, 2004; Massi *et al*. 2004). However, psychotropic effects limit the medicinal use of cannabinoids in humans. Tumor-promoting effects of cannabinoids on glioblastoma cells have also been reported (Hart *et al*. 2004). Furthermore, it is not clear to what extent human astrocytoma and glioma cells express the different cannabinoid receptors and contribute to the effects.

Therefore, we investigated the expression of CB1 and CB2 in surgical astrocytomas of WHO (World Health Organization) grades I–III, in gliomas (astrocytomas WHO IV) as well as in different glioma cell lines on the mRNA and protein level, and measured their relative contribution on the inhibition of cyclic AMP accumulation and proliferation/apoptosis by the use of agonists. We show that mean CB1 expression is slightly increased in astrocytomas and gliomas, but is not related to the WHO grade, whereas mean CB2 expression is low and similar to normal brain. In glioma cell lines CB1 is constantly expressed, but with considerable variation, whereas CB2 expression is low or absent. In

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Abbreviations used: CB, cannabinoid receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; WHO, World Health Organization.

normal brain, CB1 is located on astroglial cells, whereas in gliomas it is found on malignant and non-malignant astroglial cells. In contrast, CB2 is found on microglial and rarely on astroglial cells in normal and malignant brain tissue. In accordance with this receptor subtype expression *in situ*, agonists selective for CB1 or active on both subtypes reduced elevated cyclic AMP levels and proliferation, but did not induce apoptosis of glioma cells *in vitro*.

Materials and methods

Forskolin was purchased from Sigma (Deisenhofen, Germany). Cannabinoid agonists (HU 210, WIN 55,212–2 mesylate, JWH 133) were obtained from TOCRIS bioscience (Ellisville, MO, USA). Human CB1 and CB2 rabbit polyclonal antibodies (PA1-743 and PA1-744) were purchased from Affinity BioReagents (Golden, CO, USA).

Cell culture and tissue samples

Solid human astrocytomas WHO grades I–III, gliomas WHO grade IV, normal brain tissue and spleen were surgically dissected tissues or autopsy material from the Departments of Neurosurgery and Legal Medicine (Kiel, Germany) and were obtained in accordance with the Helsinki Declaration of 1975 and with approval of the ethics committee of the University of Kiel. All samples were frozen in liquid nitrogen immediately after surgical removal. The diagnosis was established by a pathologist/neuropathologist. The human glioma cell line U343 was obtained from Deutsches Krebsforschungszentrum (Heidelberg, Germany). Glioma cells from solid tumors (designated with an A) were obtained by dissociation and cultivation in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) as described previously (Feindt *et al*. 1995; Mentlein *et al*. 2001). Subcultures 3 to 10 were used for investigations. The purity of the cultures was controlled routinely by immunostaining for the cell type specific markers glial fibrillary acidic protein (GFAP, astrocytes/glioblastoma cells; antibody from Boehringer, Mannheim, Germany) and CD68 (contaminating microglial cells and macrophages; Feindt *et al*. 1998). Contaminations by *Mycoplasma* were checked by staining with bisbenzimide (Merck, Darmstadt, Germany). Preparation and culture of other cell types has been described previously (Pufe *et al*. 2003; Ludwig *et al*. 2005).

Real-time RT-PCR

RNA was isolated with the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), digested by DNase (65°C for 10 min; Promega, Madison, WI, USA), and cDNA synthesized with RevertAid^{TM} H Minus M-muLV Reverse Transcriptase (Fermentas, Hanover, MD, USA). Real-time PCR was performed in three replicates of each sample using an ABI 7700 Prism Sequence Detection System and TaqMan primer probes (assays on demand; Applied Biosystems, Foster City, CA, USA) using a total reactive volume of 20 μL, which contained 1 μL of 20 × Target Assay Mix, 10 μL of 2 × TaqMan Universal Master Mix and 100 or 10 ng of cDNA template (diluted in RNase-free water to 9 μL). After 10 min at 95°C for polymerase activation, 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension) were run. Glyceraldehyde-3-phosphate dehydrogenase was used as intrinsic positive control and normalizer. Cycle of threshold (C_T) of each tumor sample was averaged, and ΔC_T values = C_TCB − $C_T$glyceraldehyde 3-phosphate dehydrogenase calculated. For each gene, logarithmic linear dependence of C_T-values from the numbers of copies was verified by using different amounts of cDNA; one magnitude yielded a ΔC_T of 3.33 ($2^{3.33} = 10$).

Western blots

For protein isolation, cultivated cells were quickly rinsed twice with thermostatted 0.14 NaCl 10 mM HEPES buffer, pH 7.4, lysed with ice-cold, hypotonic 5 mM HEPES buffer, pH 7.4, and scraped off the dishes using a rubber policeman (in the case of cultivated cells). Solid tissues were homogenized with ice-cold, hypotonic 5 mM HEPES buffer, pH 7.4 for 3–5 min using a blender. All lysates were sonicated (20 s), and after addition of 1.4 M NaCl 200 mM HEPES, pH 7.4 nuclei and debris were removed by centrifugation. After protein determination, aliquots with equal protein contents (5 μg) were boiled and diluted in 50–200 μL sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, proteins separated on 10% standard SDS-PAGE, transferred onto a polyvinylidene difluoride membranes that were blocked with 5% casein for 1 h. The blots were incubated with anti-CB1 or anti-CB2 (1 : 100 for CB1, 1 : 500 for CB2) followed (after washings) by horseradish peroxidase-labeled anti-rabbit IgG (1 : 20 000; DAKO, Glostrup, Denmark). Bound antibody was visualized by enhanced chemiluminescence (ECL system; GE Healthcare, Freiburg, Germany). For semiquantitative analysis, the same samples were separated by another gel electrophoresis, and stained with Coomassie blue for total protein amount.

Immunohistochemistry

Cryostat tissue sections were fixed in acetone/methanol (1 : 1) at −20°C, washed with Tris-buffered saline (TBS) plus 0.1% Tween 20 (TBS-T, 3×, room temperature), washed with 20%, then 70% ethanol (each 2 min), blocked with Sudan black (1% in 70% ethanol) for 10 min, rinsed with 70% ethanol until dye free, then briefly with 20% ethanol, washed with TBS-T (3×), blocked with 0.1% bovine serum albumin and 0.2% glycine in TBS (1 h), then without washing incubated in with primary antibodies in TBS at 4°C overnight: anti-CB1 or anti-CB2 (rabbit polyclonal antibodies, both 1 : 100; Affinity Bioreagents; Golden, CO, USA), monoclonal anti-GFAP, monoclonal anti-Ki-67 (clone MIB-1) or, monoclonal anti-CD68 (all from DAKO, 1 : 100). After washing with TBS-T (3×), slides were incubated with fluorescent secondary antibodies (Alexa Fluor 488-labeled goat anti-rabbit IgG and Alexa Fluor 555-labeled goat anti-mouse IgG; Molecular Probes, Eugene, OR, USA; 1 : 1000, 37°C, 1 h), washed with TBS-T, TBS (2×), nuclei stained with diamidino-2-phenylindole (Molecular Probes; 1 : 30 000, 30 min room temperature), washed with TBS (3×) and finally distilled water. After embedding in Immu-Mount (Shandon, Pittsburgh, PA, USA) sections were analyzed by confocal microscopy (Zeiss, Oberkochen, Germany).

Determination of cyclic AMP concentrations

For cyclic AMP measurements, 100 000 cells grown in Petri dishes for 3 days were washed twice with thermostatted phosphate-buffered saline (PBS). Solid tumor material was cut into

small pieces of the same size and washed with PBS. Cultivated cells and solid tumor materials were exposed for 2 min at 37°C to forskolin (50 μM) alone or in combination with CB1/CB2 agonists (HU 210: 0.3 nM; JWH 133: 16 nM; WIN 55,212–2: 50 nM) in 2 mL of 37°C-thermostatted incubation buffer consisting of 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM glucose, and 20 mM HEPES, pH 7.4. After removal of the incubation mixture, cells/solid tumor material were lysed with 700 μL ice-cold 10 mM HEPES buffer, pH 7.4, supplemented with 1 mM 3-isobutyl-methyl-xanthine (IBMX, Sigma), and scraped off the culture dish/homogenized with a blender. The homogenate was mixed thoroughly; 200 μL was used for protein determination (modified Bradford assay), 500 μL was mixed with 1 mL ice-cold ethanol, centrifuged (12 000 × *g*, 5 min), and the supernatant lyophilized. The lyophilized material was reconstituted with 100 μL water, and aliquots assayed for cyclic AMP using a commercial radio-immunoassay.

Apoptotic assays

Cultivated glioblastoma cells (1×10^6) were grown for 3 days in 10% FCS-supplemented DMEM, washed in 37°C-thermostatted 0.5% FCS-supplemented DMEM, and stimulated in the same medium with 10 μg/mL camptothecin (Sigma), 0.3 nM HU 210, 16 nM JWH 133, and 50 nM WIN 55,212–2. In parallel, a control group without stimulation was used. For detection of caspase 3, the samples were washed in PBS after incubation for 2 h and incubated in 200 μL lysis buffer (1 × dithiothreitol). After lysis and following centrifugation, the supernatant was collected for further detection. The activation of the caspase-3 was measured by using the Caspase 3 Activity Assay kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The concentrations of caspase-3 were determined in relation to the protein content of the samples.

Proliferation assays

Cultivated glioblastoma cells (1×10^5 cells per 25 cm^2 dish) were grown to 60% confluence in culture medium containing 10% FCS. After changing culture medium with 0.5% FCS for 24 h, cells were stimulated for another 24 h with CB1/CB2 agonists (HU 210: 0.3 nM; JWH 133: 16 nM; WIN 55,212–2: 50 nM) in DMEM supplemented with 10% FCS or 0.5% FCS + 10 ng/mL basic fibroblast growth factor. The cells were then washed with PBS, and DNA determined fluorometrically (CyQUANT Kit, Molecular Probes) as described (Mentlein *et al*. 2001).

Results

Expression of cannabinoid receptors in solid astrocytomas/gliomas

In an initial step, we monitored the expression of CB1 and CB2 by quantitative (real time) RT-PCR in surgical tumor samples of different WHO grades (Fig. 1). Generally, CB1 expression was much higher (lower ΔC_T values correspond to higher expression) than that of CB2, which in some samples was even undetectable. CB1 expression in normal brain tissue and gliomas was comparable to that in spleen, whereas CB2 expression was 4 times higher. Although there was no statistically significant difference compared to normal brain tissue, mean CB1 expression was somewhat, but not statistically significant, elevated in astrocytomas and gliomas (about 3 ΔC_T values, corresponding to 10-fold maximum). However, there was no increase with the grade of malignancy. Mean CB2 expression in normal tissue did not differ

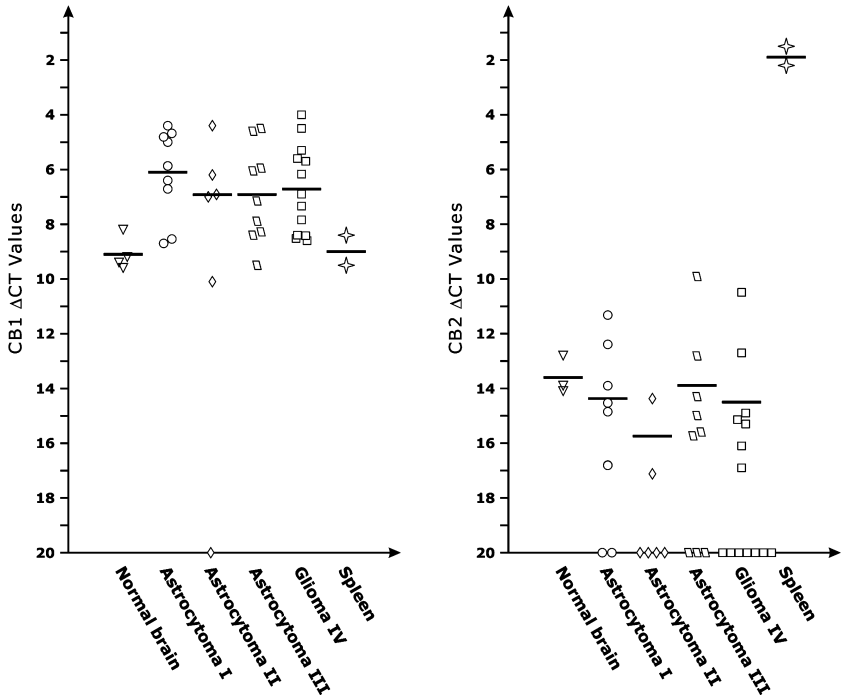

Fig. 1 Expression of cannabinoid receptors (CB) in solid astrocytomas of different WHO grades, gliomas and normal brain tissue as determined by quantitative RT-PCR. RNA was isolated from surgical samples, reverse transcribed and CB1 and CB2 quantified by specific TaqMan primers probes. ΔC_T values to glyceraldehyde 3-phosphate dehydrogenase. $\Delta C_T = 3.33$ corresponds to one magnitude. CB1 expression is higher (ΔC_T values lower) than CB2 expression, which in some samples is undetectable . Mean CB1 values are not significantly higher than that of normal tissue, but individual values of some patients are increased 10–100-fold. Spleen is shown as a positive expression control for CB2. The individual values are means of triplicate determinations with low SD (cf. Fig. 4).

from that in CB2-positive astrocytomas and gliomas. However, several tumor samples were CB2 negative.

The presence of CB1 and – with lower intensity – CB2 proteins in solid astrocytomas and gliomas was verified by western blots (Fig. 2). Strong bands of CB1 and faint ones for CB2 corresponded to molecular masses of about 60 kDa for the reduced denatured proteins.

Immunohistochemistry showed a strong expression of both receptors throughout normal brain and tumor samples (Fig. 3). CB2 staining was generally lower than CB1; however, no quantitative immunohistochemical analyses were performed. To identify CB-positive cell types, costaining was performed with a cytoskeletal marker for astrocytes (GFAP; glioma cells are often, but not necessarily positive for this protein), a nuclear proliferation marker (MIB or Ki67, most tumor cells), and a lysosomal marker for (activated) microglial cells/macrophages (CD68, KI-M6).

In normal brain sections (Fig. 3, top), CB1 staining was mainly, but not exclusively, found on GFAP-positive astroglial cells (staining merges in confocal microscopy), not on CD68-positive microglial cells. In contrast, CB2 staining was rarely found on astroglial cells, but was observed on CD68-positive microglial cells.

In glioma sections (Fig. 3, bottom), CB1 was frequently found in GFAP-positive glioma or astroglial cells (staining merges in confocal microscopy), less often on MIB-positive proliferating malignant cells, and almost not on CD68-positive microglial cells/macrophages. CB2 staining was almost not detected on GFAP-positive astroglial/glioma cells, not seen on MIB-positive proliferating tumor cells, but CD68-positive macrophages/microglial cells were often colabeled with CB2 (staining merges partially in confocal microscopy). Generally, CB1 and CB2 expression was rarely seen on proliferating cells or in areas with a high proportion of proliferating (MIB-positive) cells.

Expression of cannabinoid receptors in glioma cells *in vitro*

Surgical astrocytomas and other gliomas are heterogeneous tissues and contain, besides the tumor cells, mainly macrophages and endothelial cells. Therefore, we used cultivated glioma cell lines (U343) and glioma cells purified from solid tumors by subcultivation (A739, 764, 767, 772, 776) to quantify CB1/CB2 expression in the tumor cells (Fig. 4). Again, expression of CB2 was much lower (ΔC_T values higher) than for CB1, and in three of seven glioma cells lines tested it was undetectable. As compared to other cell types, expression of CB1 and CB2 in gliomas was lower than in the monocyte cell line THP-1 or neurinoma cells, but comparable or higher than in endothelial cells, fibroblasts or chondrocytes. As compared to solid tumors, expression of CB1 and CB2 in cultivated glioma cells was mostly lower. Proteins for CB1 and CB2 could again be detected by western blots (Fig. 2), showing strong bands of CB1 and faint ones for CB2.

In conclusion, CB1 expression is somewhat higher in gliomas compared to normal brain, but the variation is statistically insignificant. In gliomas, the receptor is located on malignant cells and other cell types. In contrast, CB2 expression is generally much lower and in gliomas does not differ from normal brain. In normal and malignant tissues, CB2 is found on micro- and astroglial cells.

Functionality of CB1 and CB2 receptors in astrocytomas and glioma cells

In the next step, we evaluated whether CB in astrocytomas and cultivated glioma cells could induce signal transduction and yielded cellular responses after stimulation with receptor subtype unselective or selective agonists.

For determining effects on signal transduction, we stimulated fresh surgical astrocytomas and gliomas (which contain intact malignant and other cells as well as debris) and cultivated glioma cells with forskolin alone or in combination with CB agonists and measured cyclic AMP-formation (Fig. 5). As expected, the adenylate cyclase activator forskolin raised cyclic AMP levels, whereas CB agonists alone showed no basal effect. All CB agonists reduced forskolin-induced cyclic AMP formation, both in solid tissue samples and cultivated glioma cells. There were no large differences between the nonselective agonist (WIN 55,212–2) and the

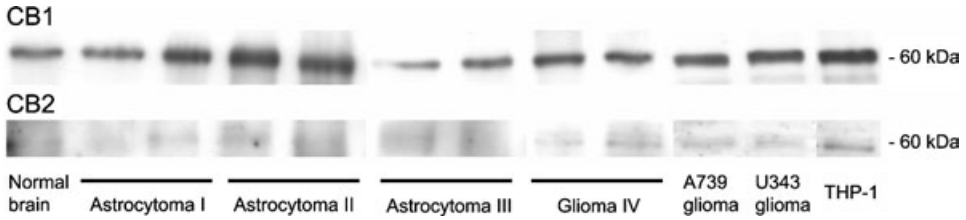

Fig. 2 Western blot of cannabinoid receptors (CB) in solid astrocytomas, solid gliomas, glioma cell lines and normal brain tissue. Protein was isolated from different samples, aliquots with the equal protein amounts were separated on SDS-PAGE, transferred on polyvinylidene difluoride membranes, incubated with specific antibodies to human CB1 or CB2 and visualized by enhanced chemiluminescence. Strong bands of CB1 and faint ones for CB2 could be detected corresponding to molecular masses of about 60 kDa for the reduced denatured proteins. This way only a qualitative detection of CB1 and CB2 protein amounts in different samples could be obtained.

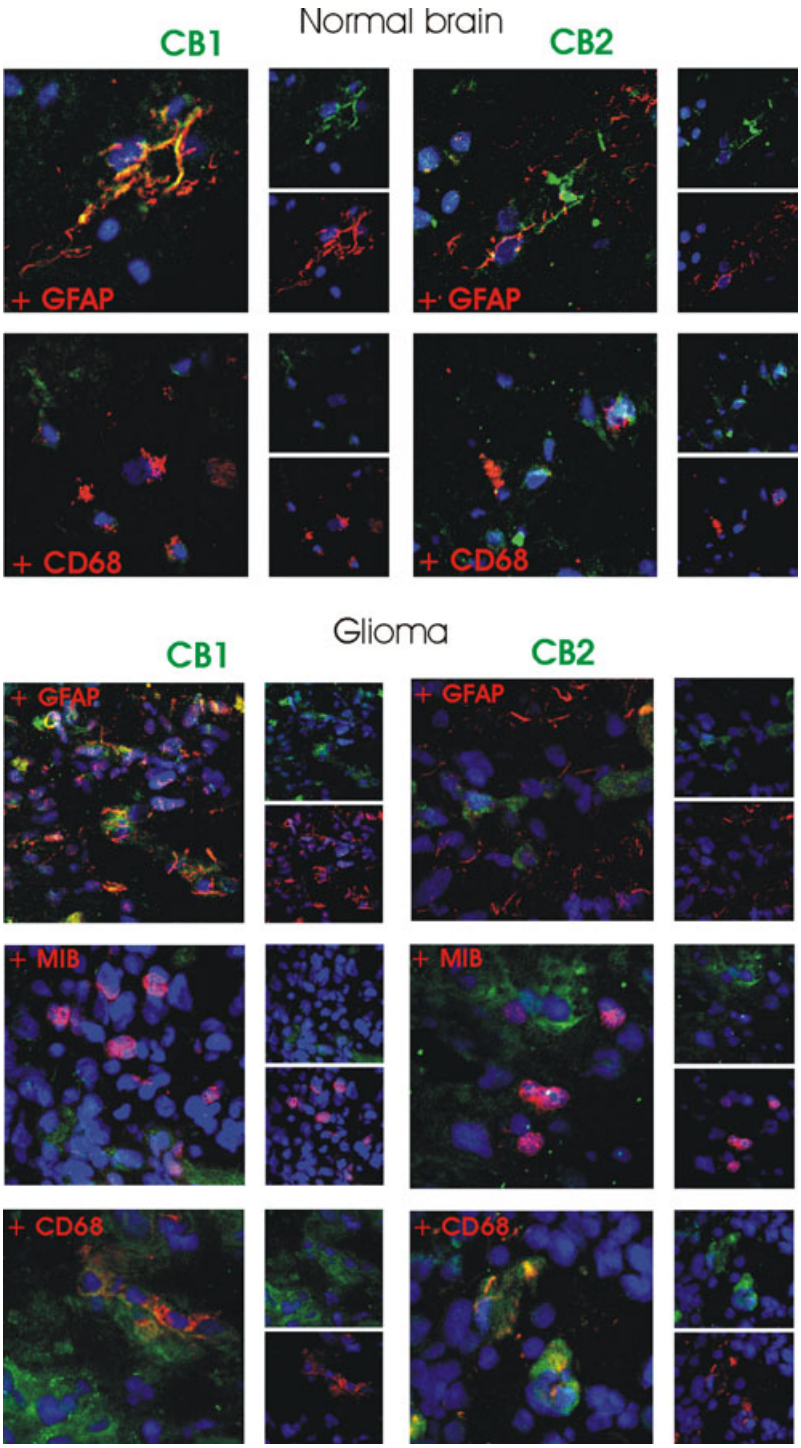

Fig. 3 Immunohistochemistry (examples of confocal microscopy) of cannabinoid receptors (CB; green, Alexa Fluor 488-labeled secondary antibody) in tissue sections (blue: nuclear counterstain) as compared to cell type-specific markers (red, Alexa Fluor 555-labeled secondary antibody) in normal brain (top) and gliomas (bottom). CB expression is found throughout the samples with varying intensities, CB2 more faintly than CB1. In normal brain, CB1 was often detected on GFAP-positive astroglial cells (yellow merge of colors) and on other cell types; however, it was never detected on CD68-positive microglial cells. CB2 was not detected on GFAP-positive astroglial cells (colors do not merge), but often on CD68-positive microglial cells (colors partly merge). In gliomas, CB1 was localized on GFAP-positive astroglial or glioma cells (yellow merge of colors); however, MIB-1-positive proliferating tumors cells were rarely labeled (colors do not merge in the example shown). CB2 was almost undetectable on GFAP-positive astroglial or glioma cells (colors only rarely merge) and undetectable on MIB-positive proliferating tumor cells (colors do not merge), but was frequently seen on CD68-positive microglial cells/macrophages (yellow merge of colors).

CB1 (HU 210) or CB2 (JWH 133) selective agonists [used at receptor-selective concentrations HU 210: 0.3 nM (0.0608 nM K_iCB1/0.524 nM K_iCB2); JWH 133: 16 nM (677 nM K_iCB1/3.4 nM K_iCB2); WIN 55,212–2: 50 nM (9.94 nM K_iCB1/16.2 nM K_iCB2). This shows that CB1 and CB2 are functionally active in malignant tissues and also in (receptor-positive) glioma cells *in vitro*.

This finding could be corroborated by the small inhibitory effects of CB agonists on the proliferation of glioma cells *in vitro* which were measured by DNA-content after 24 h (Fig. 6). At low serum concentrations in the presence of an astroglial growth factor (basic fibroblast growth factor) as well as at high serum concentrations the CB1-selective agonist HU 210 as well as the unselective agonist WIN 5512

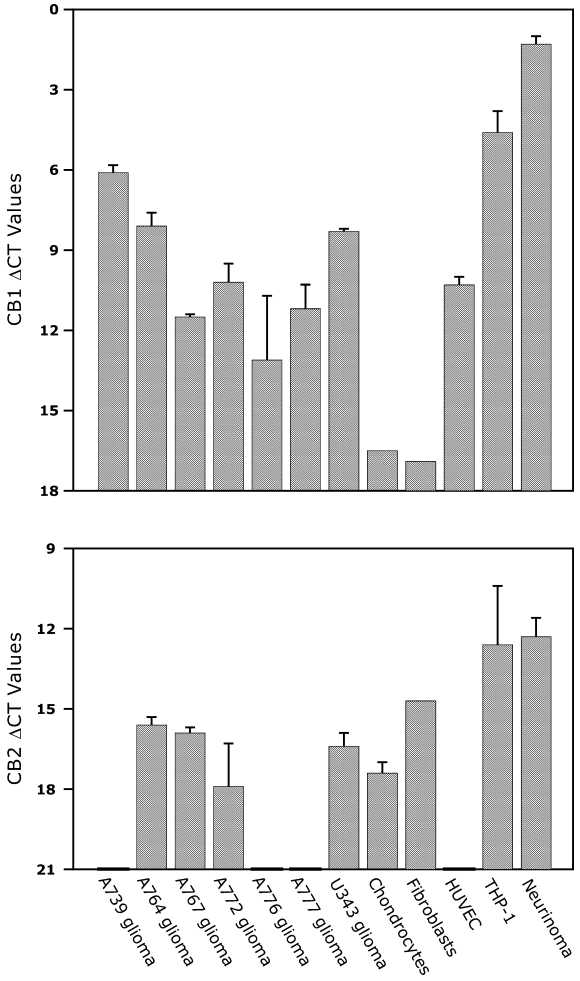

Fig. 4 Expression of cannabinoid receptors (CB) in different cell types as determined by quantitative RT-PCR, see Fig. 1. As in solid tumors, expression of CB1 and CB2 in cultivated glioma cells was mostly lower. CB1 expression was higher (ΔC_T values lower) than CB2 expression, which in some samples was undetectable. Fibroblasts, chondrocytes, human umbilical vein endothelial cells (HUVEC), THP-1 human monocytes and neurinoma cells are shown for comparison. Means ± SD of triplicate determinations.

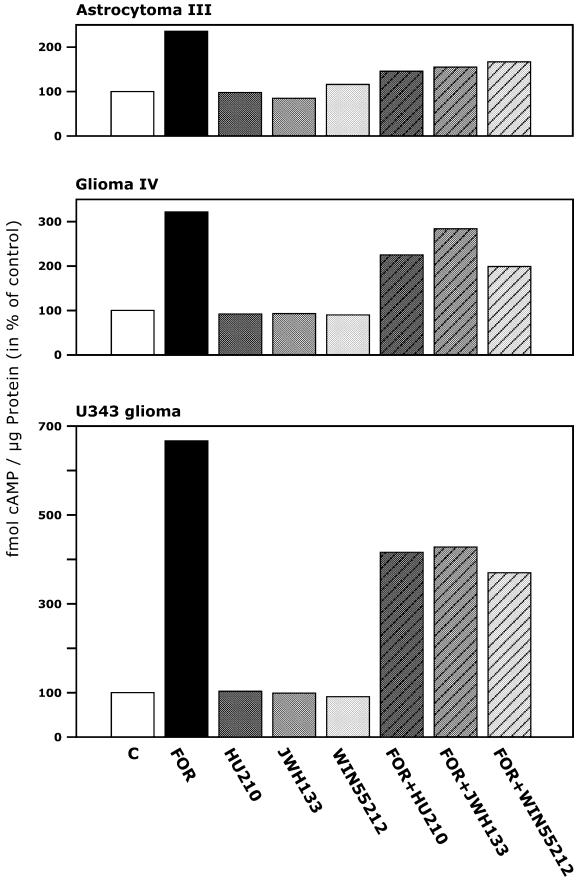

Fig. 5 Cannabinoid receptor (CB) receptor agonists reduce forskolin-elevated cyclic AMP levels in fresh astrocytoma and glioma tissue or the glioma cell line U343. Cultivated cells and solid tumor materials were challenged with forskolin (50 µM) alone or in combination with CB1/CB2 agonists (HU 210: 0.3 nM; JWH 133: 16 nM; WIN 55,212–2: 50 nM), lysed and lyophilized. Lyophilized material was reconstituted in water, and aliquots assayed for cyclic AMP using a commercial radioimmunoassay. cyclic AMP amounts were calculated in relation to protein content of different samples. The adenylate cyclase activator forskolin (FOR) raised cyclic AMP levels, whereas CB agonists alone showed no basal effect. All CB agonists reduced forskolin-induced cyclic AMP formation, both in solid tissue samples and in cultivated glioma cells. There were no large differences between the nonselective agonist (WIN 55,212–2) and the CB1 (HU 210) or CB2 (JWH 133) selective agonists.

slightly reduced cell proliferation, whereas the CB2-agonist exerted practically no effect. This result is in accordance with a high expression of CB1 and a negligible expression of CB2 on astroglial/glioma cells.

To exclude the possibility that lower DNA content after exposure to CB-agonists is mediated by apoptotic effects, the activity of the effector caspase-3 was determined in corresponding experiments. Caspase-3 activity could scarcely be detected after 2–24 h (about 620 ± 7 nmol/mg protein, at the detection limit of the assay). As a positive control for inducing apoptosis, we used the topoisomerase inhibitor camptothecin (at 10 µg/mL) which raised caspase-3 activity to 778 ± 22 nmol/mg protein after 2 h. However, none of the agonists (at the concentrations given above, 2–24 h) increased caspase-3 activity significantly (activities between 590 and 640 nmol/mg protein).

Discussion

Cannabinoids exert palliative effects in patients with cancer (e.g. inhibition of chemotherapy-induced nausea and vomiting, appetite stimulation and pain reduction) and inhibit tumor growth in laboratory animals (Guzman 2003). Effects on growth arrest and death of tumor cells as well as inhibition

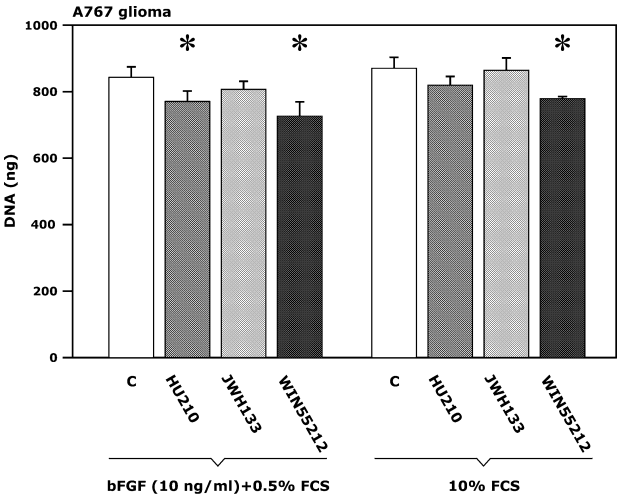

Fig. 6 Cannabinoid receptor (CB) agonists have small effects on glioma cell proliferation *in vitro*. Cells were stimulated for 24 h with CB1/CB2 agonists (HU 210: 0.3 nM; JWH 133: 16 nM; WIN 55,212–2: 50 nM) in medium supplemented with 0.5% FCS plus 10 ng/mL basic fibroblast growth factor or 10% FCS, and DNA was determined fluorometrically. CB1-selective (HU 210) and non-subtype-selective (WIN 55,212–2) agonists inhibited DNA accumulation slightly (* $p < 0.05$), whereas the CB2-selective agonist JWH 133 showed no effect. Means ± SD of four individual dishes with triple measurements each.

of tumor angiogenesis and metastasis have been reported, some also in animal glioma models (Galve-Roperh *et al*. 2000; Recht *et al*. 2001; Sanchez *et al*. 2001; Blazquez *et al*. 2003, 2004; Vaccani *et al*. 2005). Consequently, cannabinoids have been proposed for the therapy for gliomas in humans (Velasco *et al*. 2004). However, little is known about cannabinoid receptor expression in human astrocytomas and gliomas and the contribution of the different receptor subtypes. Furthermore, some cannabinoid effects appear to be mediated by other types of receptors or other cannabinoid receptor-independent mechanisms (Curran *et al*. 2005; Vaccani *et al*. 2005). Therefore, we quantified cannabinoid receptor expression in astrocytomas of different malignancy, in gliomas *in situ* and in glioma cells *in vitro*.

By quantitative RT-PCR, western blot and confocal immunohistochemistry we were able to show that CB1 is the most important receptor subtype in human astrocytoma tissue and in glioma cells, whereas the receptor subtype CB2 is low in astrocytoma tissue and absent or low in glioma cells. In glioma tissue CB1 was mainly detected on astroglial cells/GFAP-positive glioma cells, however, not frequently on proliferating malignant cells. In contrast, CB2 was mainly found on microglial cells/macrophages. In normal brain tissue the situation was similar, but also other cell types were labelled. This finding is in accordance with the normal CB1 and CB2 expression in non-malignant central and peripheral tissues: CB1 is the dominant receptor in the brain whereas CB2 is expressed in the immune system. CB1 receptors occur mainly on astroglial cells and neurons (Howlett *et al*. 2002; Stella 2004), and CB2 mainly on microglial cells (Carrier *et al*. 2004; Nunez *et al*. 2004). As solid astrocytomas and gliomas as well as malignant glial cells contain microglial cells (up to 30% of all cells; Badie and Schartner 2001) and endothelial cells, CB2 in solid tumors can be well located on tumor microglia cells.

CB1 expression in astrocytoma and glioma tissues was variable, but on average was not significantly higher than in normal brain tissues, and was unrelated to the grade of malignancy. Furthermore, glioma cells *in vitro* do not show a considerably higher CB1 expression than other cell types, e.g. endothelial cells. CB2 were detected at low levels in all solid astrocytomas, but not in all glioma cell lines. CB2 expression in astrocytoma and glioma tissues did not differ from normal brain tissue, and was much lower than in spleen. From these findings, we could not verify the results of Sanchez *et al*. (2001), which showed that in biopsies from human astrocytomas the extent of CB2 expression was directly related to tumor malignancy. Also, the monocyte cell line THP-1 shows a considerable higher CB2 expression than the positive glioma cells. Therefore, it is very likely that microglial/macrophage infiltration in human astrocytomas is responsible for a major part of the CB2 in solid gliomas. Cannabinoid therapy in humans would potentially not target tumor-elevated receptors, and these are not exclusively located on malignant cells. Consequently, complex effects can be expected.

It should be noted that our results were obtained from human tissues and cells. Glioma models in animals may behave differently. This may explain why tumor-reducing effects of cannabinoids observed in laboratory animals cannot be completely reproduced in glioma cells in culture. Although CB agonists potently reduced elevated cyclic AMP levels, lower antiproliferative effects were seen under the assay conditions tested. It may well be that antiproliferative effects on glioma cells can be observed under different culture/stimulation conditions and especially as a result of a more complex interaction *in vivo*. These may involve antiangiogenic actions or microglial/immuno reactions, which could also cause diverse side-effects (besides those on other brain cells).

In conclusion, we show that CB1 is the major cannabinoid receptor in human astrocytomas and gliomas, but is not considerably elevated as compared to normal tissue or other cell types. CB2 is a minor receptor on human astroglial tumor cells, and in solid human astrocytomas/gliomas other cell types may contribute to its low level of expression. Therefore, cannabinoid therapy of human astrocytomas and gliomas may target various cell types, is complex and should be considered with care concerning potential side-effects.

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