Cannabinoids Inhibit Glioma Cell Invasion by Down-regulating Matrix Metalloproteinase-2 Expression

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Abstract

Cannabinoids, the active components of *Cannabis sativa* L. and their derivatives, inhibit tumor growth in laboratory animals by inducing apoptosis of tumor cells and impairing tumor angiogenesis. It has also been reported that these compounds inhibit tumor cell spreading, but the molecular targets of this cannabinoid action remain elusive. Here, we evaluated the effect of cannabinoids on matrix metalloproteinase (MMP) expression and its effect on tumor cell invasion. Local administration of Δ^9-tetrahydrocannabinol (THC), the major active ingredient of cannabis, down-regulated MMP-2 expression in gliomas generated in mice, as determined by Western blot, immunofluorescence, and real-time quantitative PCR analyses. This cannabinoid-induced inhibition of MMP-2 expression in gliomas (*a*) was MMP-2–selective, as levels of other MMP family members were unaffected; (*b*) was mimicked by JWH-133, a CB_2 cannabinoid receptor–selective agonist that is devoid of psychoactive side effects; (*c*) was abrogated by fumonisin B1, a selective inhibitor of ceramide biosynthesis; and (*d*) was also evident in two patients with recurrent glioblastoma multiforme. THC inhibited MMP-2 expression and cell invasion in cultured glioma cells. Manipulation of MMP-2 expression by RNA interference and cDNA overexpression experiments proved that down-regulation of this MMP plays a critical role in THC-mediated inhibition of cell invasion. Cannabinoid-induced inhibition of MMP-2 expression and cell invasion was prevented by blocking ceramide biosynthesis and by knocking-down the expression of the stress protein p8. As MMP-2 up-regulation is associated with high progression and poor prognosis of gliomas and many other tumors, MMP-2 down-regulation constitutes a new hallmark of cannabinoid antitumoral activity. [Cancer Res 2008;68(6):1945–52]

Introduction

Cannabinoids, the active components of *Cannabis sativa* L. (marijuana) and their derivatives, exert a wide array of effects by activating specific receptors that are normally engaged by a family of endogenous ligands—the endocannabinoids (1, 2). Cannabis preparations have been used in medicine for centuries, and nowadays, there is a renaissance in the study of their therapeutic effects (3, 4). Specifically, cannabinoids have been known to exert palliative effects in patients with cancer since the early 1970s. The best established of these effects is the inhibition of chemotherapy-induced nausea and vomiting, and so capsules of Δ^9-tetrahydrocannabinol (THC), the major active component of cannabis, and its synthetic analogue nabilone are currently approved for that purpose (5, 6). In addition, several clinical trials are testing other potential palliative properties of cannabinoids in oncology such as appetite stimulation and pain inhibition (5, 6). Besides these palliative actions, cannabinoids have been proposed as potential antitumoral agents owing to their ability to inhibit the growth and angiogenesis of various types of tumor xenografts in animal models (5). Studies on malignant gliomas and other models of cancer strongly support the conclusion that cannabinoids decrease tumor progression by at least two mechanisms: the apoptotic death of tumor cells (7–10) and the inhibition of tumor angiogenesis (8, 11–14). It has also been reported that cannabinoids inhibit tumor cell migration and spreading (12, 15–18). However, the molecular targets of this cannabinoid effect remain elusive. Among the different factors involved in the acquisition of invasive capacities by tumor cells, the action of matrix metalloproteinases (MMP) plays a pivotal role. MMPs have long been linked to tumor invasion owing to their crucial involvement in the breakdown of the extracellular matrix and in the proteolytic activation of various classes of tumor progression factors. Accordingly, increased expression and activation of MMPs are found in almost every type of human cancer compared with normal tissue, and this has been associated with poor patient prognosis (19–21). This background prompted us to study the effect of cannabinoid administration on MMP expression by tumor cells and its effect on tumor cell invasion. Here, we report that cannabinoid administration inhibits MMP-2 expression in cultured glioma cells, in mice bearing gliomas, and in two patients with glioblastoma multiforme. We also show that down-regulation of MMP-2 expression underlies cannabinoid-induced inhibition of glioma cell invasion and is mediated by the sphingolipid ceramide and the stress protein p8, two key signaling elements of cannabinoid antitumoral action (10).

Materials and Methods

Cannabinoids. The cannabinoid agonists THC, JWH-133, and anandamide were kindly given by Alfredo Dupetit (The Health Concept, Richelbach, Germany), John W. Huffman (Department of Chemistry, Clemson University, South Carolina), and Daniele Piomelli (University of California, Irvine, Irvine CA), respectively. The cannabinoid antagonists SR141716 and SR144528 were kindly given by Sanofi-Aventis (Montpellier, France). For *in vitro* incubations, ligands were directly applied at a final DMSO concentration of 0.1% to 0.2% (v/v). For *in vivo* administration to mice, cannabinoids were prepared at 1% (v/v) DMSO in 100 μL of PBS supplemented with 5 mg/mL of bovine serum albumin. No significant influence of the vehicle was observed on any of the variables determined. The preparation of THC for administration to patients is described below.

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

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doi:10.1158/0008-5472.CAN-07-5176

Cell culture. The rat C6.9 and C6.4 glioma cell lines were cultured in Ham's F12 medium supplemented with 10% FCS. The human SW1088, T98 G, U87 MG, and U118 MG astrocytoma cell lines were cultured in DMEM supplemented with 10% FCS. Twenty-four hours before the experiments, cells were transferred to their respective serum-free media. Human umbilical vein endothelial cells (11) and rat cortical astrocytes (10) were isolated and cultured as described before.

Cell invasion assay. Cell invasion was monitored by using cell culture inserts (BD Biosciences). After exposure to different stimuli, cells were trypsinized, washed, resuspended in DMEM, and loaded into the insert. DMEM supplemented with 2.5% fetal bovine serum was placed in the plate well as a cell migration stimulus. Cells were allowed to migrate for 4 h (C6.9 and C6.4 cells) or 6 h (U87 MG cells) at 37°C through an 8-μm polyethylene terephthalate track-etched membrane that had been precoated with Matrigel basement membrane matrix (BD Biosciences) at 9.6 mg/mL and subsequently blocked with 1% bovine serum albumin in PBS. Then, cells from the upper side of the membrane were removed, and the remaining cells on the bottom side of the membrane were fixed with 70% ethanol, stained with crystal violet, and counted.

Tumor generation in mice. Tumors were generated in immunodeficient mice by subcutaneous flank inoculation of 5×10^6 C6.9 or C6.4 glioma cells in 100 μL of PBS supplemented with 0.1% glucose. When tumors reached a volume of 300 to 400 mm^3, animals were assigned randomly to the various groups and injected peritumorally (at ~2 mm from the tumor) for 8 days with 500 μg/d of THC, 50 μg/d of JWH-133, and/or 60 μg/d of fumonisin B1 (Alexis). Control animals were injected with vehicle. Tumors were measured with an external caliper and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Human tumor samples. Tumor biopsies were obtained from two recurrent patients with glioblastoma multiforme who had been treated with THC. The characteristics of the patients and the clinical study have been described in detail elsewhere (22). Briefly, each day, an aliquot of THC (100 mg/mL in ethanol) was dissolved in 30 mL of physiologic saline solution supplemented with 0.5% (w/v) human serum albumin and the resulting solution was administered intratumorally to the patients. Patient 1 received a total of 1.46 mg of THC for 30 days, whereas patient 2 received a total of 1.29 mg of THC for 26 days. Samples were either frozen (for Western blotting) or fixed in formalin and embedded in paraffin (for immunomicroscopy).

Western blot analysis. Particulate tissue fractions were subjected to SDS-PAGE, and proteins were transferred from the gels onto polyvinylidene difluoride membranes. The blots were incubated with antibodies raised against different MMPs and α-tubulin was used as a loading control (Supplementary Table S1). In all cases, samples were subjected to

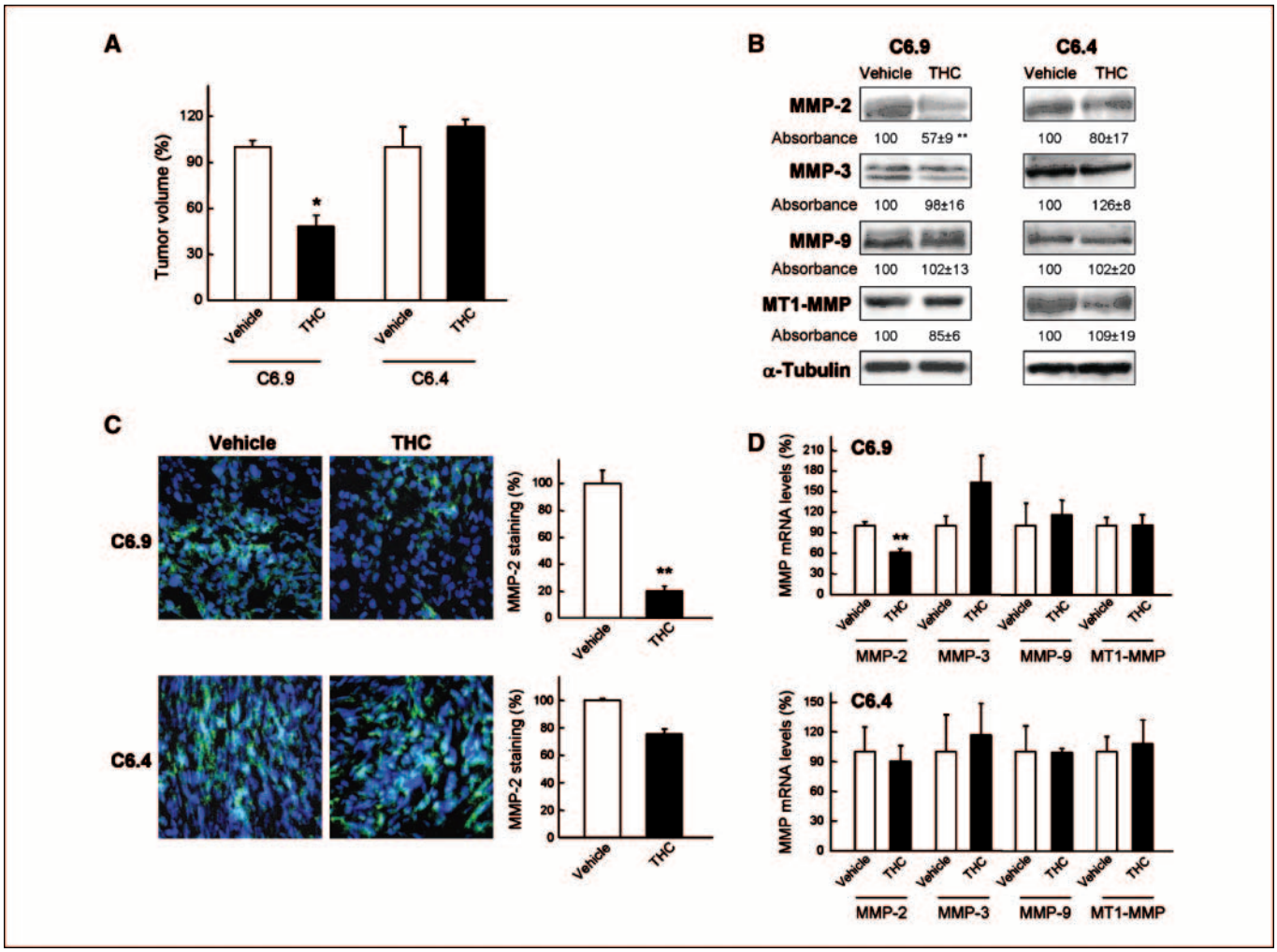

Figure 1. THC inhibits MMP-2 expression in mouse gliomas. Mice bearing tumors generated by inoculation of C6.9 or C6.4 glioma cells were treated with either vehicle or THC for 8 days. *A,* tumor volume at the end of the treatment. *B,* MMP-2, MMP-3, MMP-9, and MT1-MMP expression as determined by Western blot. Absorbance values relative to those of α-tubulin are given in arbitrary units. *C,* MMP-2 expression as determined by immunofluorescence microscopy. *Green,* MMP-2; *blue,* cell nuclei. Relative values of MMP-2 pixels per cell nucleus are given. *D,* MMP-2, MMP-3, MMP-9, and MT1-MMP mRNA levels as determined by real-time quantitative PCR. Significantly different (**, $P < 0.01$; *, $P < 0.05$) from control animals (n = 3–4 for each experimental group).

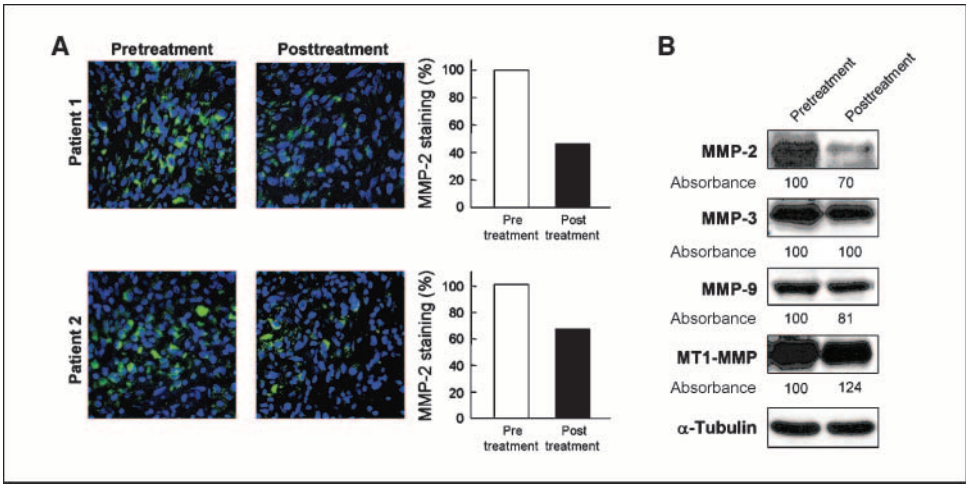

Figure 2. THC inhibits MMP-2 expression in two patients with glioblastoma multiforme. Patients were subjected to THC administration as described in Materials and Methods. *A,* MMP-2 expression as determined by immunofluorescence microscopy. *Green,* MMP-2; *blue,* cell nuclei. Relative values of MMP-2 pixels per cell nucleus are given. *B,* MMP-2, MMP-3, MMP-9, and MT1-MMP expression in the tumor of patient 1 before and after THC treatment as determined by Western blot. Absorbance values relative to those of loading controls (*α-Tubulin*) are given in arbitrary units.

luminography with an enhanced chemiluminescence detection kit (Amersham Life Sciences). Densitometric analysis of the blots was done with Kodak Molecular Imaging Software 4.0 in a Kodak Image Station 4000 MM.

Immunofluorescence microscopy. Mouse tumors were dissected and frozen, and sections were fixed in acetone for 10 min. Human tumors were fixed in 10% buffered formalin and then paraffin-embedded. Sections (5 μm) were stained with anti-rat MMP-2 (1:200; Torrey Pines Biolabs) or anti–MMP-2 (1:200; Chemicon) antibody as described (13). Sections were mounted with Mowiol mounting medium (Merck) containing TOTO-3 iodide (1:1,000; Molecular Probes) to stain cell nuclei. Fluorescence images were acquired using Metamorph-Offline 6.2 software (Universal Imaging) and Zeiss Axioplan 2 Microscope. Pixel quantification was obtained from the analysis of 5 to 10 fields chosen randomly from three to four sections per tumor. Fluorescence thresholds were set at 75 (low threshold) and 255 (high threshold).

Real-time quantitative PCR. RNA was isolated using Trizol reagent (Invitrogen) including a DNase digestion step using the Real Star Kit (Durviz). cDNA was obtained with Transcriptor Reverse Transcriptase (Roche Applied Science). Real-time quantitative PCR assays were done using the FastStart Master Mix with Rox (Roche Applied Science) and probes were obtained from the Universal Probe Library Set (Roche Applied Science). The primers used are shown in Supplementary Table S2. Amplifications were run in a 7900 HT-Fast Real-Time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA levels as reference.

RNA interference. U87 MG astrocytoma cells were transfected with small interfering RNA (siRNA) duplexes using the DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's instructions. Four different double-stranded RNA duplexes corresponding to hMMP2, hMMP3, hMMP9, and hMMP14 (ON-TARGETplus SMARTpool L-005959-00-0010, L-005968-00, L-005970-00, and L-004145-00, respectively) and double-stranded RNA duplexes corresponding to hEGR1 (VELGA-000007) were from Dharmacon. Double-stranded RNA duplexes corresponding to human p8 (5′-GGAGGACCCAGGACAGGAU-3′) and a nontargeted control (5′-UUCUCCGAACGUGUCACGU-3′) were from Eurogentec. Double-stranded RNA corresponding to hATF3 was purchased from Roche Applied Science; it was obtained from the esiWay Resource RZPDp3000C1214D and then synthesized using the X-tremeGENE siRNA Dicer Kit (Roche Applied Science). Transfection efficiency was >80%, as monitored by using a control fluorescent siRNA (siGLO RISC-Free siRNA D-001600-01-20; Dharmacon).

MMP-2 overexpression. pbluescript KS-hMMP2 was kindly provided by Barry L. Marmer (Department of Medicine, Washington University School of Medicine, St. Louis, MO). hMMP2 cDNA was obtained from this construct by PCR to add an *Eco*RI site at 5′ (5′-TTAGAATTCATGGAGG-CGCTAATGGCCCGG-3′) and a *Not*I site at 3′ (5′-ATTGCGGCCGCTCAG-CAGCCTAGCCAGTCGGAT-3′), and was subsequently cloned into a pCDNA.4 vector for eukaryotic expression. U87MG astrocytoma cells were transfected with the MMP-2–expressing plasmid or the empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were trypsinized and seeded at a density of 5,000 cells/cm^2. Cells were transferred to a serum-free medium 18 h before performing the different treatments. Transfection efficiency was >70% as monitored by using a green fluorescent protein–expressing plasmid.

ELISA. MMP-2 protein levels were determined in cell culture medium by using the human MMP-2 Biotrack ELISA System (Amersham).

Statistics. The results shown represent mean ± SD. Statistical analysis was performed by ANOVA with a post hoc analysis using the Student-Neuman-Keuls test or by unpaired Student's *t* test.

Results

THC inhibits MMP-2 expression in mouse gliomas. To test whether cannabinoid administration affects MMP levels, we generated subcutaneous gliomas in mice. The specificity of cannabinoid action was ascertained by the parallel study of C6.9 and C6.4 glioma cells, which constitute well-established models of cannabinoid-responsive and cannabinoid-resistant cells, respectively (7, 10, 23). Tumors were treated with either vehicle or THC and MMP levels were determined by Western blot. Cannabinoid administration decreased tumor growth (Fig. 1*A*) and MMP-2 expression (Fig. 1*B*) in C6.9-cell gliomas. The effect seemed to be MMP family member–selective as THC did not reduce the expression of MMP-3, MMP-9, and MT1-MMP (MMP-14), which have been also associated with glioma invasion and progression (24–26). The levels of other MMPs (MMP-1, MMP-8, MMP-10, and MMP-13) remained unchanged as well (Supplementary Fig. S1*A*). In contrast with C6.9-cell gliomas, neither tumor growth (Fig. 1*A*) nor MMP-2 expression (Fig. 1*B*; Supplementary Fig. S1*A*) were affected by THC treatment in C6.4-cell gliomas. Immunofluorescence microscopy experiments confirmed the cannabinoid-induced decrease of MMP-2 levels in the cannabinoid-sensitive but not in the cannabinoid-resistant tumors (Fig. 1*C*). Evaluation of MMP mRNA levels by real-time quantitative PCR provided further evidence for the selective down-regulation of MMP-2 in THC-treated C6.9-cell gliomas (Fig. 1*D*).

THC inhibits MMP-2 expression in two patients with glioblastoma multiforme. To obtain further support for the potential therapeutic value of cannabinoid-induced down-regulation of MMP-2 expression, we analyzed tumor samples from two

patients with recurrent glioblastoma multiforme (22). The patients were subjected to local THC administration and biopsies were taken before and after treatments. Immunomicroscopy analysis showed that tumor MMP-2 levels were lower after cannabinoid delivery in both patients (Fig. 2*A*). This was confirmed by Western blot analysis in tumor biopsies from patient 1, in which the levels of other MMPs remained unchanged (Fig. 2*B*; Supplementary Fig. S1*B*). Unfortunately, we were unable to obtain appropriate samples for Western blotting from patient 2.

The CB_2 receptor agonist JWH-133 inhibits MMP-2 expression in mouse gliomas. Because cannabinoid-based therapeutic strategies should be as devoid as possible of psychotropic effects, which are mediated by brain CB_1 receptors (3, 4), and gliomas express functional CB_2 receptors (7), which are not linked to cannabinoid psychoactivity, we gave mice JWH-133, a CB_2 receptor-selective agonist that exerts antitumoral activity without overt psychoactive side effects (27). JWH-133 decreased tumor growth (Fig. 3*A*) and MMP-2 expression in glioma xenografts, as determined by both Western blot (Fig. 3*B*) and immunomicroscopy analyses (Fig. 3*C*). The levels of the other MMPs remained unchanged upon JWH-133 treatment (Fig. 3*B*).

The ceramide biosynthesis inhibitor fumonisin B1 prevents JWH-133–induced MMP-2 down-regulation in mouse gliomas. The sphingolipid messenger ceramide has been implicated in the regulation of tumor growth and angiogenesis by cannabinoids (7, 10, 13). The involvement of ceramide in cannabinoid-induced inhibition of MMP-2 expression was thus tested by the use of fumonisin B1, a selective inhibitor of sphingolipid biosynthesis that is widely used to depress ceramide levels *in vivo* and that prevents cannabinoid-induced ceramide accumulation in glioma cells (13). Fumonisin B1 abrogated the inhibitory action of JWH-133 on tumor growth (Fig. 3*A*). In addition, the decrease in MMP-2 levels induced by cannabinoid administration was prevented by cotreatment of the animals with fumonisin B1, as determined by both Western blot (Fig. 3*B*) and immunomicroscopy analyses (Fig. 3*C*).

THC inhibits MMP-2 expression in cultured glioma cells. To test whether the cannabinoid-induced down-regulation of MMP-2 expression observed *in vivo* reflects the direct effect of cannabinoids on tumor cells, we conducted cell culture experiments. The cannabinoid did not significantly affect cell viability throughout the time interval in which MMP-2 determinations were performed (data not shown). In line with the aforementioned *in vivo* observations, THC decreased MMP-2 mRNA levels in cultures of C6.9 cells but not of C6.4 cells (Fig. 4*A*). Cannabinoid-induced inhibition of MMP-2 expression was also evident in a number of human glioma cell lines (astrocytomas SW1088, T98 G, U87 MG,

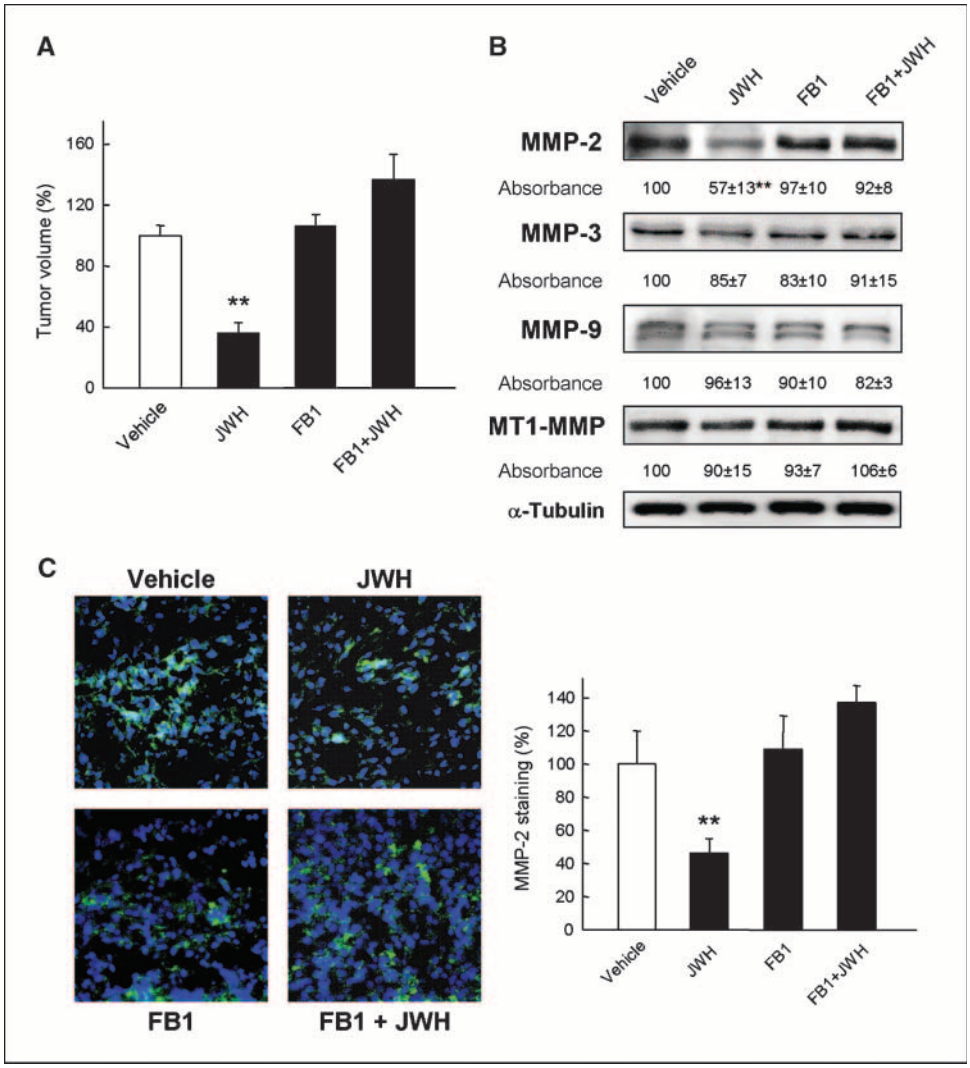

Figure 3. Effect of JWH-133 and fumonisin B1 on MMP-2 expression in mouse gliomas. Mice bearing C6.9-cell gliomas were treated with vehicle, JWH-133 (*JWH*), fumonisin B1 (*FB1*), or JWH-133 plus fumonisin B1 (*FB1+JWH*) for 8 days. *A,* tumor volume at the end of the treatment. *B,* MMP-2, MMP-3, MMP-9, and MT1-MMP expression as determined by Western blot. Absorbance values relative to those of α-tubulin are given in arbitrary units. *C,* MMP-2 expression as determined by immunofluorescence microscopy. *Green,* MMP-2; *blue,* cell nuclei. Relative values of MMP-2 pixels per cell nucleus are given. One representative tumor of each experimental group is shown. Significantly different (**, $P < 0.01$) from control animals ($n = 3$–4 for each experimental group).

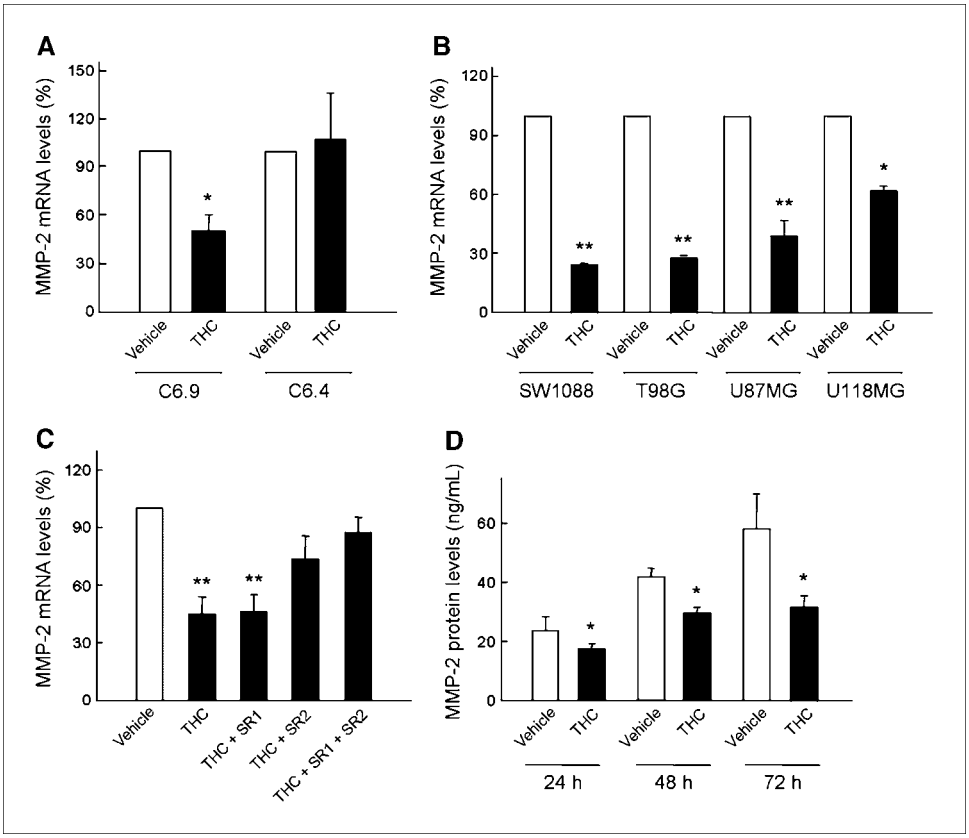

Figure 4. THC inhibits MMP-2 expression in cultured glioma cells. *A* to *C*, C6.9 and C6.4 glioma cells (*A*; $n = 4$); SW1088, T98 G, U87 MG, and U118 MG astrocytoma cells (*B*; $n = 2$–3); or U87-MG cells (*C*; $n = 3$) were cultured for 24 h with vehicle (*open columns*) or 1.5 μmol/L of THC alone or plus 1 μmol/L of SR141716 (*SR1*) and/or 1 μmol/L of SR144528 (*SR2*; *closed columns*). MMP-2 mRNA levels were determined by real-time quantitative PCR. *D*, U87 MG cells were cultured for 24, 48, or 72 h with vehicle (*open columns*) or 1.5 μmol/L of THC (*closed columns*) and MMP-2 protein levels in the culture medium were determined by ELISA ($n = 3$). Significantly different from the respective vehicle incubations (**, $P < 0.01$; *, $P < 0.05$).

and U118 MG; Fig. 4*B*), but not in primary cultures of normal, nontransformed rat astroglial cells (data not shown). The use of the CB_1-selective antagonist SR141716 and the CB_2-selective antagonist SR144528 supported the assumption that THC acts on U87 MG cells mostly via CB_2 receptors, although the contribution of CB_1 receptors should not be ruled out (Fig. 4*C*). ELISA determinations showed that THC also decreased MMP-2 protein levels in U87 MG cells (Fig. 4*D*). However, challenge with THC at 1.5 μmol/L for 24, 48, or 72 hours did not significantly affect MMP-2 levels in human umbilical vascular endothelial cell cultures (data not shown), in contrast with a previous report showing decreased MMP-2 activity upon exposure of these cells to the endocannabinoid analogue, 2-methyl-2′-*F*-anandamide at 10 μmol/L for 24 hours (14). We tested the effect of the natural endocannabinoid anandamide in our U87 MG-cell system and found that this compound was highly cytotoxic at 10 μmol/L for 24 hours. At 5 μmol/L for 24 hours, it did not affect cell viability and decreased MMP-2 expression to 68 ± 19% of vehicle-treated cells ($n = 3$; $P < 0.05$), but this effect was not prevented by SR141716 or SR144528, either alone or in combination (data not shown), pointing to a CB receptor–independent action.

MMP-2 down-regulation is involved in THC-induced inhibition of glioma cell invasion. As MMP-2 is involved in the acquisition of highly invasive characteristics by different types of tumor cells, including glioma cells (19, 25, 28), we next examined whether cannabinoids modulate glioma cell invasion. THC inhibited the invasion of C6.9 and U87 MG cells but not of C6.4 cells (Fig. 5*A*). Of interest, both cannabinoid-sensitive and cannabinoid-resistant cells were equally affected by the broad-spectrum MMP inhibitor GM6001 and the MMP-2/MMP-9–selective inhibitor 5 (Fig. 5*A*; ref. 29). To further test the involvement of MMP-2 in THC-mediated depression of glioma cell invasion, we manipulated cellular MMP-2 mRNA levels. On the one hand, transfection of U87 MG cells with a MMP-2–directed siRNA (which diminished MMP-2 mRNA levels to 26 ± 11% of control siRNA-transfected cells; $n = 3$, $P < 0.05$) decreased basal cell invasion and abrogated THC-inhibited cell invasion (Fig. 5*B, left*). On the other hand, transfection of U87 MG cells with a MMP-2–expressing vector (which increased MMP-2 mRNA levels to 236 ± 42% of control vector–transfected cells; $n = 3$, $P < 0.01$) slightly—although not significantly—stimulated basal cell invasion and abrogated THC-inhibited cell invasion (Fig. 5*B, right*). The selective involvement of MMP-2 down-regulation in the action of THC was supported by the observation that transfection of U87 MG cells with either a MMP-3–directed siRNA (which diminished MMP-3 mRNA levels to 12 ± 6% of control siRNA-transfected cells; $n = 2$, $P < 0.01$), a MMP-9–directed siRNA (which diminished MMP-9 mRNA from detectable down to nondetectable levels; $n = 2$), or a MMP-14–directed siRNA (which diminished MT1-MMP mRNA levels to 64 ± 2% of control siRNA-transfected cells; $n = 2$, $P < 0.05$) did not significantly affect basal cell invasion or the inhibitory action of THC on cell invasion (Fig. 5*C*).

THC inhibits MMP-2 expression and invasion in cultured glioma cells via ceramide and p8. We next asked how THC signals for inhibiting MMP-2 expression and glioma cell invasion. The effect of THC on MMP-2 expression and invasion (Fig. 6*A*) in U87 MG cells was prevented by ISP-1, a selective inhibitor of ceramide synthesis *de novo* that blocks THC-induced ceramide accumulation in glioma cells (10). We have previously reported that the stress protein p8 is a ceramide effector in cannabinoid-induced

apoptosis (10). To determine the role of p8 in the action of THC, we selectively reduced p8 expression by RNA interference. Thus, transfection of U87 MG cells with a p8-directed siRNA (which diminished p8 mRNA levels to 32 ± 17% of control siRNA-transfected cells; $n = 4$, $P < 0.01$) abrogated the inhibitory action of THC on MMP-2 expression and cell invasion (Fig. 6*B*).

The early-expression gene *EGR-1* (also designated as krox-24) up-regulates the expression of ATF-3 (30), which in turn, represses the *MMP-2* gene (31–33). As cannabinoids have been shown to induce EGR-1 expression (34, 35), we examined the possible involvement of the EGR-1/ATF-3 pathway in THC-mediated MMP-2 down-regulation. However, transfection of U87 MG cells with either an EGR-1–directed siRNA (which diminished EGR-1 mRNA levels to 44 ± 12% of control siRNA-transfected cells; $n = 4$, $P < 0.05$) or an ATF-3–directed siRNA (which diminished ATF-3 mRNA levels to 23 ± 3% of control siRNA-transfected cells; $n = 3$, $P < 0.01$) did not significantly affect the inhibitory action of THC on MMP-2 expression and cell invasion (Fig. 6*C* and *D*).

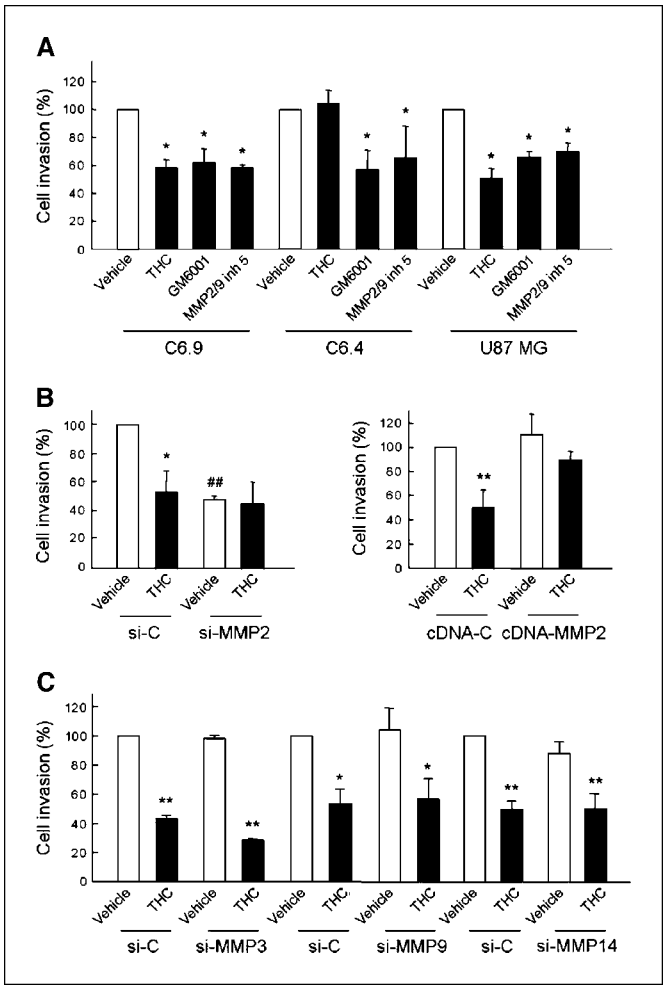

Figure 5. MMP-2 down-regulation is involved in THC-induced inhibition of glioma cell invasion. *A,* C6.9, C6.4, and U87 MG cells were cultured for 24 h with vehicle (*open columns*), 1.5 μmol/L of THC, 10 μmol/L of GM6001, or 10 μmol/L of MMP-2/MMP-9–selective inhibitor 5 (*closed columns*). The MMP inhibitors were maintained in the cell medium during the invasion assays. *B,* U87 MG cells were transfected with a control (*si-C*) or MMP-2–directed siRNA (*si-MMP2*; *left*) or with a control (*cDNA-C*) or MMP-2 cDNA (*cDNA-MMP2*; *right*) and cultured for 24 h with vehicle (*open columns*) or 1.5 μmol/L of THC (*closed columns*). *C,* U87 MG cells were transfected with the respective control siRNAs (*si-C*) or with siRNAs directed against MMP-3, MMP-9, or MMP-14 and cultured for 24 h with vehicle (*open columns*) or 1.5 μmol/L of THC (*closed columns*). Cell invasion was subsequently determined ($n = 2$). Significantly different from the respective vehicle incubations (**, $P < 0.01$; *, $P < 0.05$) or from the respective vehicle-treated si-C–transfected cells ($^{\#\#}$, $P < 0.01$).

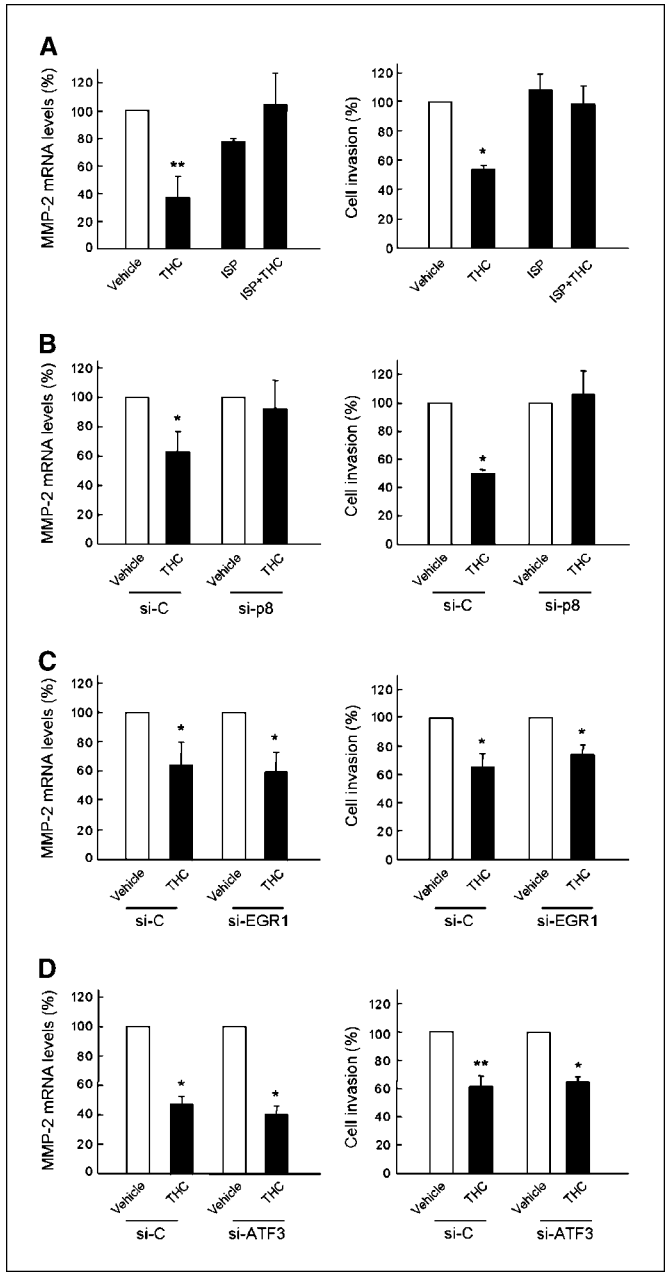

Figure 6. THC inhibits MMP-2 expression and invasion in cultured glioma cells via ceramide and p8. *A,* U87 MG cells were cultured for 24 h with vehicle (*open columns*), 1.5 μmol/L of THC, 1.5 μmol/L of ISP-1, or 1.5 μmol/L of THC plus 1.5 μmol/L of ISP-1 (*closed columns*). *B* to *D,* U87 MG cells were transfected with the respective control siRNAs (*si-C*) or with siRNAs directed against p8 (*B*), EGR1 (*C*), or ATF3 (*D*) and cultured for 24 h with vehicle (*open columns*) or 1.5 μmol/L of THC (*closed columns*). MMP-2 mRNA levels (real-time quantitative PCR; *left*) as well as cell invasion (*right*) were subsequently determined ($n = 3$–4). Significantly different (**, $P < 0.01$; *, $P < 0.05$) from the respective vehicle incubations.

Discussion

Despite the widely described antitumoral activity of cannabinoids in various animal models of cancer (5), the molecular

effectors of these actions have not been fully characterized as yet. Here, we show that cannabinoid administration selectively down-regulates MMP-2 expression in mice bearing gliomas as well as in two patients with recurrent glioblastoma multiforme. Cannabinoid-induced inhibition of MMP-2 expression was also evident in cultured glioma cells, indicating that the changes observed in gliomas *in vivo* reflect—at least in part—the direct effect of cannabinoids on tumor cells. MMP-2 expression is up-regulated in almost all human cancers, including gliomas, and this has been shown to be closely associated with negative prognosis (19, 21, 28). Additionally, experiments in animal models of cancer—including glioma—in which tumor cells express decreased or increased levels of MMP-2 have provided direct evidence for a role of MMP-2 in tumor progression (19, 21, 25, 26). Likewise, data in the present report shows that inhibition of MMP-2 expression mediates cannabinoid-induced inhibition of glioma cell invasion and supports MMP-2 down-regulation as a new hallmark of cannabinoid antitumoral activity. Nonetheless, we are aware that the role of MMPs in tumor progression is an extremely complex issue. Thus, accruing basic and clinical evidence supports the assumption that MMPs and other proteases could either enhance or suppress tumor progression depending on factors such as the type of enzyme and tumor, and taking into consideration the characteristics of the experimental setting (21, 36). In addition, tissue inhibitors of metalloproteinases may have a dual role in tumor progression via their classical MMP inhibitory actions (19, 20), and by recently discovered MMP-independent actions such as the promotion of tumor proliferation and angiogenesis (37). Hence, in addition to the down-regulation of MMP-2 expression, factors such as changes in tissue inhibitor of metalloproteinase-1 levels (17), are most likely involved in both cannabinoid-induced inhibition of MMP-2 enzymatic activity (11) and the overall control of tumor cell invasion by cannabinoids (present report).

It has been shown that cannabinoids modulate sphingolipid-metabolizing pathways, thereby increasing the intracellular levels of ceramide (5, 7), a lipid second messenger that inhibits tumor cell growth and survival in different systems (38). Specifically, the stimulation of ceramide synthesis *de novo* is critically involved in cannabinoid-induced apoptosis of glioma cells (10, 23) and inhibition of glioma angiogenesis (13). The findings reported here expand the role of *de novo*–synthesized ceramide in cannabinoid antitumoral action and support the hypothesis that this lipid messenger, via the stress-related protein p8, is involved in the regulation of MMP-2 expression and tumor cell invasion. In the context of the "sphingolipid rheostat" theory, the antiproliferative sphingolipid ceramide would blunt MMP-2 expression as well as tumor growth and invasion (present study, refs. 5, 13), whereas the mitogenic sphingolipid sphingosine 1-phosphate would shift the balance towards MMP-2 up-regulation (39) and tumorigenesis (38).

The use of cannabinoids in medicine is limited by the psychoactive effects mediated by neuronal CB_1 receptors (1, 2). Although these adverse effects are within the range of those accepted for other medications, especially in cancer treatment, and tend to disappear with tolerance upon continuous use, it is obvious that cannabinoid-based therapies devoid of side effects would be desirable (3–5). Because glioma cells express functional CB_2 receptors (7), we tested the effect of the nonpsychoactive, CB_2 receptor-selective agonist JWH-133 and found that it indeed depresses MMP-2 expression *in vivo*. Likewise, the use of CB receptor type–selective antagonists indicates that CB_2 receptors participate in THC-induced inhibition of MMP-2 expression in glioma cells. As selective CB_2 receptor activation to mice has been shown to inhibit the growth and angiogenesis of gliomas (11, 13, 27), skin carcinomas (8) and melanomas (15), our observations further support the possibility of finding cannabinoid-based antitumoral strategies devoid of nondesired psychotropic side effects.

Acknowledgments

Received 9/5/2007; revised 11/27/2007; accepted 12/3/2007.

Grant support: Grants from Ministerio de Educación y Ciencia (SAF2006-00918, M. Guzmán), Comunidad de Madrid (S-SAL/0261/2006 and 950344, M. Guzmán), and Universidad Complutense de Madrid (PR1/07-14896, C. Blázquez). M. Salazar is a recipient of a Ph.D. fellowship from Ministerio de Educación y Ciencia. A. Carracedo was a recipient of a fellowship from Consejería de Educación del Gobierno Vasco. M. Lorente is a recipient of a Juan de la Cierva contract from Ministerio de Educación y Ciencia.

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We are indebted to Eva Resel, Sofía Torres, and Sonia Hernández for expert technical assistance, and to the rest of the members of the lab for discussion and advice.

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