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Down-regulation of tissue inhibitor of metalloproteinases-1 in gliomas: a new marker of cannabinoid antitumoral activity?

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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 inhibiting tumor angiogenesis. It has also been reported that cannabinoids inhibit tumor cell invasiveness, but the molecular targets of this cannabinoid action remain elusive. Here we evaluated the effects of cannabinoids on the expression of tissue inhibitors of metalloproteinases (TIMPs), which play critical roles in the acquisition of migrating and invasive capacities by tumor cells. Local administration of Δ^9-tetrahydrocannabinol (THC), the major active ingredient of cannabis, down-regulated TIMP-1 expression in mice bearing subcutaneous gliomas, as determined by Western blot and immunofluorescence analyses. This cannabinoid-induced inhibition of TIMP-1 expression in gliomas (i) was mimicked by JWH-133, a selective CB_2 cannabinoid receptor agonist that is devoid of psychoactive side effects, (ii) was abrogated by fumonisin B1, a selective inhibitor of ceramide synthesis *de novo*, and (iii) was also evident in two patients with recurrent glioblastoma multiforme (grade IV astrocytoma). THC also depressed TIMP-1 expression in cultures of various human glioma cell lines as well as in primary tumor cells obtained from a glioblastoma multiforme patient. This action was prevented by pharmacological blockade of ceramide biosynthesis and by knocking-down the expression of the stress protein p8. As TIMP-1 up-regulation is associated with high malignancy and negative prognosis of numerous cancers, TIMP-1 down-regulation may be a hallmark of cannabinoid-induced inhibition of glioma progression.

Keywords: Cannabinoid; Tissue inhibitor of metalloproteinases; Glioma; Invasion; Ceramide; Experimental therapeutics

1. 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 (Howlett et al., 2002; Piomelli, 2003). Cannabis preparations have been used in medicine for centuries, and nowadays there is a renaissance in the study of their therapeutic effects (Di Marzo and Petrocellis, 2006; Mackie, 2006). Specifically, cannabinoids have been known to exert palliative effects in cancer patients since the early 1970s. The best established of these effects is the inhibition of chemotherapy-induced nausea and vomiting, and nowadays capsules of Δ^9-tetrahydrocannabinol (THC), the major active component of cannabis, and its synthetic analogue nabilone are approved for that purpose (Guzmán, 2003; Hall et al., 2005). In addition, several clinical trials are testing other potential palliative properties of cannabinoids in oncology such as appetite stimulation and pain inhibition (Guzmán, 2003; Hall et al., 2005). 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 (Guzmán, 2003). Studies on malignant brain tumors (gliomas) and other models of cancer strongly support that cannabinoids decrease tumor progression by at least two mechanisms: the apoptotic death

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of tumor cells (Galve-Roperh et al., 2000; Casanova et al., 2003; Carracedo et al., 2006a,b) and the inhibition of tumor angiogenesis (Blázquez et al., 2003, 2004, 2006; Casanova et al., 2003; Portella et al., 2003; Pisanti et al., 2007). It has also been reported that cannabinoids inhibit the migration and spreading of tumor cells (Portella et al., 2003; Blázquez et al., 2006; Grimaldi et al., 2006). However, the molecular targets of this cannabinoid effect remain elusive. Among the various factors involved in the acquisition of migrating and invasive capacities by cancer cells, the concerted action of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases – TIMPs) plays a pivotal role. MMPs have been long linked to cancer cell invasion owing to their crucial involvement in extracellular matrix breakdown. Accordingly, increased expression and activation of MMPs is found in almost all human cancers compared with normal tissue, and this increase has been associated with poor patient prognosis (Egeblad and Werb, 2002; Deryugina and Quigley, 2006; Overall and Kleifeld, 2006). Likewise, cannabinoid administration down-regulates MMP-2 expression in vascular endothelial and tumor cells (Blázquez et al., 2003; Pisanti et al., 2007). In addition to the MMPs, one of the most prominent MMP inhibitors, TIMP-1, is selectively up-regulated in numerous cancers, and this has been shown to be closely associated with negative prognosis, making TIMP-1 a promising candidate for new negative-prognosis marker (Hornebeck et al., 2005; Würtz et al., 2005; Yasui et al., 2005; Chirco et al., 2006). This finding may be explained, at least in part, by MMP-independent actions of TIMP-1 such as promotion of tumor cell proliferation and survival as well as of tumor angiogenesis (Hornebeck et al., 2005; Chirco et al., 2006). This background prompted us to explore the effect of cannabinoid administration on TIMP-1 expression by cancer cells. Here we report that cannabinoid administration inhibits TIMP-1 expression in cultured glioma cells, in mice bearing gliomas and in two patients with glioblastoma multiforme. In addition, our data support that this effect is mediated by the sphingolipid ceramide and the stress protein p8, two key signaling elements of cannabinoid antitumoral action (Guzmán, 2003; Blázquez et al., 2004; Carracedo et al., 2006a,b).

2. Methods

2.1. Cannabinoids

THC and JWH-133 were kindly given by Alfredo Dupetit (The Health Concept, Richelbach, Germany) and J.W. Huffman (Department of Chemistry, Clemson University, SC), respectively. For *in vitro* incubations cannabinoids were directly applied at a final DMSO concentration of 0.1–0.2% (v/v). For *in vivo* administration to mice cannabinoids were prepared at 1% (v/v) DMSO in 100 µl PBS supplemented with 5 mg/ml bovine serum albumin. No significant influence of the vehicle was observed on any of the parameters determined. The preparation of THC for administration to patients is described below.

2.2. Cell culture

The rat C6.9 and C6.4 glioma cell lines were cultured in Ham's F12 medium supplemented with 10% fetal calf serum. The human SW1088, T98 G, U87 MG and U118 MG astrocytoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Twenty-four hours before the experiments, cells were transferred to their respective serum-free DMEM media. Primary human glioma cells were prepared from a glioblastoma multiforme (=grade IV astrocytoma). The biopsy was digested with collagenase (type Ia; Sigma, St. Luois, MO) in DMEM at 37 °C for 90 min and the supernatant was seeded in DMEM containing 15% fetal calf serum and 1 mM glutamine. Cells were kept in primary culture for about 2 weeks, subsequently seeded for the experiments, and finally transferred to 0.5%-serum DMEM 24 h before cannabinoid addition. Cell viability was determined by the MTT test.

2.3. Tumor generation in mice

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

2.4. Human tumor samples

Tumor biopsies were obtained from two recurrent glioblastoma multiforme patients who had been treated with THC. The characteristics of the patients and the clinical study have been described in detail elsewhere (Blázquez et al., 2004; Guzmán et al., 2006). Briefly, each day an aliquot of THC (100 mg/ml in ethanol) was dissolved in 30 ml of physiological 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, while Patient 2 received a total of 1.29 mg of THC for 26 days (Guzmán et al., 2006). Samples were either frozen (for Western blotting) or fixed in formalin and embedded in paraffin (for immunomicroscopy).

2.5. Western blot analysis

Particulate tissue fractions were subjected to SDS-PAGE, and proteins were transferred from the gels onto polyvinylidene fluoridefluoride membranes. The blots were incubated with antibodies raised against TIMP-1 (1:200; Chemicon, Temecula, CA), TIMP-2 (1:1000, Chemicon) or TIMP-3 (1:1000; Affinity BioReagents, Golden, CO). α-Tubulin (1:4000, Sigma) was used as a loading control. In all cases, samples were subjected to luminography with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL). Densitometric analysis of the blots was performed with Kodak Molecular Imaging Software 4.0 in a Kodak Image Station 4000 MM (Rochester, NY).

2.6. 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-TIMP-1 antibody (1:50; Chemicon) as described previously (Blázquez et al., 2004). Sections were mounted with Mowiol mounting medium (Merck, Darmstadt, Germany) containing TOTO-3 iodide (1:1000; Molecular Probes, Leyden, The Netherlands) to stain cell nuclei. Fluorescence images were acquired using Metamorph-Offline 6.2 software (Universal Imaging, Downingtown, PA) and Zeiss Axioplan 2 Microscope. Data were obtained from the analysis of five to 10 fields chosen randomly from three to four sections per tumor. Cells were routinely counted by an observer blinded to the experimental protocol, and positive cells were identified as green-stained cells with fluorescence thresholds set at 170 (low threshold) and 255 (high threshold).