Amphiregulin Is a Factor for Resistance of Glioma Cells to Cannabinoid-Induced Apoptosis

MAR LORENTE, ARKAITZ CARRACEDO, SOFÍA TORRES, FRANCESCO NATALI, AINARA EGIJA, SONIA HERNÁNDEZ-TIEDRA, MARÍA SALAZAR, CRISTINA BLÁZQUEZ, MANUEL GUZMÁN, AND GUILLERMO VELASCO

Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain

KEY WORDS
amphiregulin; DNA arrays; p8; tumor xenografts

ABSTRACT
Gliomas, one of the most malignant forms of cancer, exhibit high resistance to conventional therapies. Identification of the molecular mechanisms responsible for this resistance is therefore of great interest to improve the efficacy of the treatment against these tumors. Δ9-Tetrahydrocannabinol (THC), the major active ingredient of marijuana, and other cannabinoids inhibit tumor growth in animal models of cancer, including glioma, an effect that relies, at least in part, on the ability of these compounds to induce apoptosis of tumor cells. By analyzing the gene expression profile of two sub-clones of C6 glioma cells with different sensitivity to cannabinoid-induced apoptosis, we found a subset of genes with a marked differential expression in the two sub-clones. Furthermore, we identified the epidermal growth factor receptor ligand amphiregulin as a candidate factor to mediate the resistance of glioma cells to cannabinoid treatment. Amphiregulin was highly overexpressed in the cannabinoid-resistant cell line, in both in culture and in tumor xenografts. Moreover, in vivo silencing of amphiregulin rendered the resistant tumors xenografts sensitive to cannabinoid antitumoral action. Amphiregulin expression was associated with increased extracellular signal-regulated kinase (ERK) activation, which mediated the resistance to THC by blunting the expression of p8 and TRB3—two genes involved in cannabinoid-induced apoptosis of glioma cells. Our findings therefore identify Amphirregulin as a factor for resistance of glioma cells to THC-induced apoptosis and contribute to unraveling the molecular bases underlying the emerging notion that targeted inhibition of the EGFR pathway can improve the efficacy of antitumoral therapies. © 2009 Wiley-Liss, Inc.

INTRODUCTION
Glioblastoma multiforme (GBM), or grade IV astrocytoma, is the most frequent class of malignant primary brain tumors and one of the most aggressive forms of cancer. As a consequence, survival after diagnosis is normally just 6–12 months (Maher et al., 2001; Nieder et al., 2006; Wong et al., 2007). This dramatic behavior is mainly due to the high invasiveness and proliferation rate of GBM as well as to its high resistance to standard therapies. Current treatments based on the use of surgery, radiotherapy and chemotherapy with temozolomide, carbmustin or carboplatin have a very limited efficacy (Maher et al., 2001; Nieder et al., 2006; Wong et al., 2007). It is therefore essential to develop new strategies for the management of GBM, which will most likely require a combination of therapies to obtain significant clinical results.

The hemp plant Cannabis sativa produces approximately 70 unique compounds known as cannabinoids, of which Δ9-tetrahydrocannabinol (THC) is the best studied owing to its high potency and abundance in cannabis (Gaoni and Mechoulam, 1964). THC exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (Mechoulam et al., 1995)—that bind to and activate specific cannabinoid receptors. So far, two cannabinoid-specific G protein-coupled receptors have been cloned and characterized from mammalian tissues (Howlett et al., 2002): CB1 and CB2. The CB1 receptor is particularly abundant in discrete areas of the brain, but is also expressed in peripheral nerve terminals and various extra-neural sites. In contrast, the CB2 receptor was initially described to present in the immune system (Monro et al., 1993), although recently it has been shown that expression of this receptor also occurs in cells from other origins (Bennito et al., 2003; Carracedo et al., 2006a; Casanova et al., 2003; Galve-Roperh et al., 2000).

One of the most exciting areas of research in the cannabinoid field is the study of the potential application of cannabinoids as antitumoral agents (Guzman, 2003). Thus, cannabinoid administration has been shown to curb the growth of several models of tumor xenografts in rats and mice (Guzman, 2003), including gliomas. Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Spanish Ministry of Education and Science (MEC); Grant numbers: HP2005/0021, SAF2006/0092; Grant sponsor: Santander-Complutense; Grant number: PR84/07-15856; Grant sponsor: Comunidad de Madrid; Grant number: S-SAL/0261/2006.

Arkaitz Carracedo and Ainara Egia are currently at Cancer Genetics Program, Departments of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA.

Francesco Natali is currently at Laboratory of Biochemistry and Molecular Biology, Department of Biological and Environmental Sciences and Technologies, University of Salento, Lecce, Italy.

*Correspondence to: Guillermo Velasco, Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, c/ José Antonio Novais sn, 28040 Madrid, Spain. E-mail: gvd@bbm1.ucm.es

Received 18 September 2008; Accepted 13 January 2009

DOI 10.1002/glia.20856

Published online 00 Month 2009 in Wiley InterScience (www.interscience.wiley.com).
GLIA

TRB3 Tribbles homologue 3.

THC

GBM Glioblastoma Multiforme

ERK Extracellular signal-regulated kinase

EGFR Epidermic growth factor receptor

ER Endoplasmic reticulum

way inhibitors.

ment could be overcome by the utilization of EGFR path-

treatment. Our results also support that the resistance of
tumor vessels (Guzman, 2003) that promotes resistance of glioma cells to cannabinoid
tHERG path-

teract with cannabinoid-based antitumoral
ttreatment in order to improve the se-

ty of cannabinoid-based antitumoral therapies. Here, we show that the epidermal growth factor
(receptor (EGFR) ligand amphiregulin is a pivotal factor

cation of this pro-apoptotic pathway plays a major role in

mediating the antitumoral activity of cannabinoids (Carra-

ceto et al., 2006a,b). Based on this preclinical evidence, a

pilot clinical trial has been recently run to investigate the

antitumoral action of THC on recurrent gliomas (Guzman et al., 2006). As one of the most clinically-relevant features
of gliomas is their high resistance to chemotherapy (Maher et al., 2001), the present work was undertaken to identify

the molecular factors associated to the resistance of tumor
cells to cannabinoid treatment in order to improve the se-

lectivity and efficacy of cannabinoid-based antitumoral therapies. Here, we show that the epidermal growth factor
receptor (EGFR) ligand amphiregulin is a pivotal factor

that promotes resistance of glial cells to cannabinoid treatment. Our results also support that the resistance of

amphiregulin-overexpressing cells to cannabinoid treatment

could be overcome by the utilization of EGFR pathway inhibitors.

MATERIALS AND METHODS

Reagents

THC was from THC Pharm GmbH (Frankfurt, Germany), tyrphostin AG1478 [a selective inhibitor of
EGFR tyrosine kinase activity (Gschwind et al., 2003)] and U0126 [a selective inhibitor of ERK activation (Bain et al., 2003)] were from Calbiochem (San Diego, CA), and recombinant mouse amphiregulin was from R&D systems (Minneapolis, MN). Double-stranded RNA duplexes corresponding to rat amphiregulin were from Dharmaco (Lafayette, CO).

Cell Culture and Viability

C6.4 and C6.9 cells were cultured in complete medium as in (Galve-Roperh et al., 2000). C6.9 and C6.4 cells were originally generated by the limiting dilution

method (Davoust et al., 1998) and subsequently charac-

terized for their sensitivity to THC-induced cell death (Galve-Roperh et al., 2000). Cells were transferred to a serum-free or conditioned medium 18 h before perform-

ing the different treatments. In experiments with exoge-

nous amphiregulin, this ligand was added 2 h before

THC. Cell viability was determined after 72 h of THC
treatment by using the MTT test (Promega, Madison, WI) according to manufacturer's instructions. THC stock
solution was prepared in dimethylsulfoxide. Control
incubations contained the same amount of dimethylsul-

famide and no significant effect was observed in any of

the parameters determined throughout this study at the

final concentration used (0.1–0.2%, v/v).

Conditioned Medium Experiments

C6.4 or C6.9 cells were seeded in complete medium at a density of 30,000 cells/cm² and, after 8 h, transferred to a serum-free medium for 18 h. Media were subse-

sequently recovered, centrifuged and incubated alone,

with an anti-amphiregulin (20 μg/mL; RB-257-P1ABX, Lab Vision Corporation, Fremont, CA) or with an isotype (rabbit IgG; 20 μg/mL; Amersham Biosciences Corp., Piscataway, NJ) antibody for 1 h at 37°C. To study the influence of the conditioned media on cell viability, C6.9 cells were seeded in complete medium at a density of 10,000 cells/cm² and, after 8 h, transferred to the corre-

sponding conditioned medium 18 h before performing the different treatments. Finally, cell viability was eval-

uated as described above.

Transfections

Seventy five percent-confluent C6.4 cells were transfected with control (siC) or rat amphiregulin-selective (siAREG) siRNAs using the X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) according to manufacturer's instructions. Twenty-four hours after transfection, cells were trypsinized and seeded in complete me-

dium at a density of 10,000 cells/cm². After 8 h, cells were transferred to a serum-free medium for 18 h and the differ-

ent treatments were performed. Transfection efficiency was greater than 85% in each experiment as monitored using a control fluorescent siRNA (Qiagen, Hilden, Germany).

Reverse Transcription-PCR Analysis

RNA was isolated using the RNeasy Protect kit (Qiagen) including a DNase digestion step using the
RNase-free DNase kit (Qiagen). cDNA was subsequently obtained using Transcriptor Reverse Transcriptase (Roche). The following sense and antisense primers were used to amplify rat amphiregulin [GTGCTGGTGG ACCTTGTTTTCT and CAGGACGCGGTTATGATGC (509 bp product)], and GAPDH [GGGAGCGACTGG CATGGCTTCC and ATGTGGCCATGAGGTCCAC

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>THC</td>
<td>9-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRB3</td>
<td>Tribbles homologue 3.</td>
</tr>
</tbody>
</table>

GLIA
AMPHIREGULIN PROMOTES RESISTANCE TO THC

CAC (318 bp product). PCR reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min followed by a final extension step of 72°C for 5 min. The number of cycles (27–30 cycles for rat amphiregulin and 22–25 cycles for GAPDH) was adjusted to allow detection in the linear range. Finally, PCR products were separated on 1.5% agarose gels.

Real-Time Quantitative PCR

cDNA was obtained as described above. Real-time quantitative PCR assays were performed using the Fast-Start Master Mix with Rox (Roche) and probes were obtained from the Universal Probe Library Set (Roche). The following sense/antisense primers and probes were obtained from the Universal Probe Library Set (Roche). Start Master Mix with Rox (Roche) and probes were used for detecting rat AREG-selective siRNA per injection was administered in each tumor coinciding with days 1 and 7 of the treatment with vehicle or THC. RNA sequences to target rat AREG were obtained from Dharmacon.

Immunoprecipitation and Western Blot

Western blot analysis was performed as described (Gomez del Pulgar et al., 2002). Anti-ERK1/2, antiphosphorylated-ERK1/2 (Cell Signaling, Danvers, MA) and anti-α-tubulin (Sigma Chemical Co, St. Louis, MO) antibodies were used. For immunoprecipitation, 500 μg of cell lysate were incubated with anti-EGFR antibody (Upstate biotechnology, Singapore, Singapore) and the immunocomplex was captured by adding 10 μl of protein A sepharose beads. After washing, samples were resuspended in 2× Laemml buffer, boiled for 5 min, centrifuged and subsequently loaded on 8% SDS-PAGE gels. Finally, immunoblot analysis using anti-PY20 (BD, Franklin Lakes, NJ) or anti-EGFR antibody was performed. Densitometric analyses were carried out using Quantity One software (Biorad, Hercules, CA).

Analysis of Gene Expression in C6.9 and C6.4 Cells

Total RNA was isolated from control C6.9 and C6.4 cells, amplified, labeled and hybridized on a 22k rat array (Agilent technologies) (see Supp. Info. MIAME).

Active Caspase 3 Immunostaining

Cell cultures grown on 12-mm-coverslips were washed in PBS, fixed with 4% paraformaldehyde (20 min at room temperature), permeabilized with 0.5% Triton X100 (5 min at room temperature) and incubated with rabbit polyclonal anticleaved caspase-3 Asp175 (1:100; Cell Signaling Technology) antibody. Incubation with appropriate Alexa-488-conjugated secondary antibody (Invitrogen, Eugene, Oregon, USA) was performed in the dark at room temperature for 1 h. Cell nuclei were stained with Hoechst 33342 (Invitrogen). Finally, coverslips were mounted in ProLong Gold antifade reagent (Invitrogen) and visualized in a Leica TCS SP2 confocal microscope.

Immunostaining and TUNEL in Tumor Samples

Samples from tumors xenografts were dissected and frozen. Sections were blocked to avoid non-specific binding with 10% goat antiserum and 0.25% Triton X-100 in PBS. For phosphorylated-ERK1/2 and TRB3 immunodetection, slides were incubated with rabbit antiphosphorylated-ERK1/2 (1/250; Cell Signaling) or anti-TRB3 antibodies (1/500; Abcam, Cambridge, UK). TUNEL was performed using the in situ cell death detection kit (Roche). In all cases, sections were mounted with

Tumor Xenografts

Tumors were induced by subcutaneous injection in nude mice of 10 × 10^6 C6.9 or C6.4 cells in PBS supplemented with 0.1% glucose. When tumors had reached an average size of 250 mm^3, animals were assigned randomly to various groups and injected peritumorally for 8 days (C6.9) or 15 days (C6.4) with THC (15 mg/kg per day) or vehicle in 100 μL of PBS supplemented with 5 mg/mL defatted and dialyzed BSA. Injections with the above treatments were performed a few millimeters from the left (even days) or the right (odd days) side of each tumor. Tumors were measured with external calipers, and volume was calculated as (4π/3) × (length/2) × (width/2).

In vivo AREG silencing was performed by using a 1:1 mixture of AteloGene™ (Koken Co. Ltd., Tokyo, Japan) and 10 μM HPLC-purified double-stranded siRNA, according to manufacturer’s instructions. Briefly, the mix of Atelogene and siRNA (1 nmol of control or AREG-selective siRNA per injection) was administered in each tumor coinciding with days 1 and 7 of the treatment with vehicle or THC. RNA sequences to target rat AREG were obtained from Dharmacon.
Data on 7 selected genes were validated by real-time quantitative PCR (Table 1). In order to identify genes poten-
tially associated with cannabinoid treatment [specifically, C6.4 cells are more resistant than C6.9 cells to THC-
induced apoptosis (Carracedo et al., 2006b; Galve-
Roperh et al., 2000; Gomez del Pulgar et al., 2002) and Fig. 1A, left panel]. In order to identify genes poten-
tially associated to cannabinoid treatment, here, by using DNA arrays, we analyzed the gene expression pro-
file of both sub-clones of glioma cells. Differences in the expression of genes related to several functions, includ-
ing metabolism and cell differentiation, proliferation and survival were found between both cell sub-clones. Those
genes that were expressed with at least a difference of 5-fold between C6.9 and C6.4 cells were initially selected to further investigate their potential involvement in the resistance to cannabinoid treatment (Fig. 1A, middle panel; the complete list of genes is shown in Supp. Info. Table 1). Data on 7 selected genes were validated by using real-time quantitative PCR (Table 1).

Amphiregulin Mediates the Resistance of C6.4 Cells to THC Action

One of the genes that were more strongly up-regu-
lated in the cannabinoid-resistant C6.4 sub-clone was the heparin-bound EGF-like receptor ligand amphiregulin (Fig. 1A, right panel). Amphiregulin is synthesized as a precursor, cleaved by metalloproteinases and released in its active form to the extracellular medium (Berasain et al., 2007; Gschwind et al., 2003). Since it has been shown that amphiregulin acts as a pro-survival factor for tumor cells (Berasain et al., 2007; Gschwind et al., 2003; Johnston et al., 2006; Mishima et al., 1998), we selected this protein as a potential candidate to mediate the resistance of glioma cells to THC-induced apoptosis. Of interest, selective knock-down of amphiregulin increased the sensitivity of C6.4 cells to cannabinoid treatment but did not affect the viability or induced apoptosis of C6.4 cells by itself (Fig. 1B). We therefore investigated whether the presence of soluble amphiregulin in the medium of C6.4 cells was responsible for the resistance of these cells to THC. In agreement with this idea, conditioned medium from C6.4 cells rendered C6.9 cells resistant to THC-induced cell death, and this effect was prevented by amphiregulin neutralization with an anti-amphiregulin antibody (Fig. 1C). Moreover, addition of exogenous amphiregulin increased the resistance of C6.9 cells to THC (Fig. 1D), further confirming that amphiregulin promotes resistance to THC-induced glioma cell death.

It has been shown that amphiregulin mediates its pro-
survival actions by engaging EGFR (Gschwind et al., 2003). In agreement with this observation and our afore-
mentioned data, EGFR phosphorylation was higher in C6.4 than in C6.9 cells and addition of exogenous amphiregulin enhanced EGFR phosphorylation (Fig. 2A, left panel). Likewise, AREG silencing reduced EGFR phosphorylation of C6.4 cells (Fig. 2A, middle panel) and phosphorylation of this receptor was increased in C6.9 cells that have been incubated with the amphiregulin-enriched C6.4 conditioned medium (Fig. 2A, right panel). In order to verify that amphiregulin-induced re-
sistence to THC was mediated via activation of the EGFR, we incubated C6.9 and C6.4 cells with tyrphostin AG1478 [a pharmacological inhibitor of EGFR tyrosine kinase activity (Gschwind et al., 2003)]. Interestingly, pre-incubation with AG1478 sensitized C6.4 (Fig. 2B) but not C6.9 (Fig. 2C) cells to a further THC treatment, suggesting that EGFR over-activation is responsible for the resistance of C6.4 cells to THC. Accordingly, blockade of EGFR tyrosine kinase activity nullified the protective effect that the addition of exogenous amphiregulin exerted on C6.9 cells (Fig. 2D). These data support that the mechanism by which amphiregulin mediates the resistance to THC involving the activation of the EGFR. In line with this idea, extracellular signal-regu-
lated kinase (ERK), which is activated upon EGFR stim-
ulation, showed a higher phosphorylation level in C6.4 than in C6.9 cells (Fig. 3A, left panel), and transfection with a selective siRNA for amphiregulin reduced ERK phosphorylation in C6.4 cells (Fig. 3A, middle panel). Likewise, conditioned medium from C6.4—but not from C6.9—cells led to increased ERK phosphorylation in C6.9 cells (Fig. 3A, right panel), indicating that amphiregulin promotes ERK activation in glioma cells. Therefore, we tested whether the protective effect of amphiregulin relied on ERK activation. In agreement with this possibility, pharmacological inhibition of the ERK pathway by U0126 rendered C6.4—but not C6.9—cells more sensitive to cannabinoid treatment (Fig. 3B and C). Likewise, blockade of the ERK pathway nullified the protective effect that the addition of exogenous amphiregulin exerted on C6.9 cells (Fig. 3D). These observations strongly support that amphiregulin promotes the resist-

RESULTS

Gene Expression Profile of THC-Sensitive and Resistant C6 Glioma Cells

To investigate the molecular features associated with the resistance of tumor cells to cannabinoid treatment we used two sub-clones of C6 glioma cells, designated as C6.9 and C6.4 (Davoust et al., 1998), that exhibit a different sensitivity to cannabinoid treatment [specifically, C6.4 cells are more resistant than C6.9 cells to THC-induced apoptosis (Carracedo et al., 2006b; Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002) and Fig. 1A, left panel]. In order to identify genes potentially associated to cannabinoid resistance, here, by using DNA arrays, we analyzed the gene expression profile of both sub-clones of glioma cells. Differences in the expression of genes related to several functions, including metabolism and cell differentiation, proliferation and survival were found between both cell sub-clones. Those genes that were expressed with at least a difference of 5-fold between C6.9 and C6.4 cells were initially selected to further investigate their potential involvement in the resistance to cannabinoid treatment (Fig. 1A, middle panel; the complete list of genes is shown in Supp. Info. Table 1). Data on 7 selected genes were validated by using real-time quantitative PCR (Table 1).

Statistics

Unless otherwise specified, results shown represent mean ± S.D. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student-Neu-
man-Keuls test.
Fig. 1. Increased amphiregulin expression confers C6.4 cells resistance to THC. (A) Left panel: Effect of THC (72 h) on the viability of C6.4 (cannabinoid-resistant) and C6.9 (cannabinoid-sensitive) cells (n = 6; **P < 0.01 and *P < 0.05 from vehicle-treated cells). Middle panel: Differences in the expression of selected genes between C6.9 and C6.4 cells as determined by DNA arrays. Values are expressed as the ratio of C6.4 to C6.9 mRNA levels. Right panel: mRNA levels of amphiregulin in C6.9 and C6.4 cultured cells as determined by real-time quantitative PCR (n = 4; **P < 0.01 from C6.9 cells AREG expression). (B) Left panel: Effect of vehicle (Veh), THC (2.75 μM) or THC + exogenous AREG (100 ng/mL) on the viability of C6.4 cells transfected with control (siC) or amphiregulin selective (siAREG) siRNA (n = 8; **P < 0.01 from siC-transfected vehicle-treated cells, *P < 0.01 from siAREG-transfected vehicle-treated cells, **P < 0.01 from siAREG-transfected THC-treated cells). Inset: mRNA amphiregulin levels as determined by RT-PCR of siC and siAREG-transfected C6.4 cells. Transfection efficiency was >85%. RNA was isolated after 18 h of incubation in serum-free medium; a representative experiment is shown. Values in parentheses correspond to amphiregulin mRNA levels as determined by real-time quantitative PCR (n = 5; **P < 0.01 from siC-transfected cells; values of siC-transfected cells are set at 1.0). Right panel: Effect of THC (48 h) on apoptosis (active caspase-3 immunostaining) of C6.4 cells transfected with siC or siAREG. Values inside the photomicrographs are expressed as the percentage of active caspase-3 positive cells relative to the total number of nuclei in each field (mean ± S.D.; n = 3; **P < 0.01 from the corresponding siC or siAREG-transfected vehicle-treated cells, **P < 0.01 from siC-transfected THC-treated cells) (C) C6.9 cells were incubated for 18 h in the corresponding conditioned medium (CM), treated with THC and, after 72 h, cell viability was determined (n = 5; **P < 0.01 and *P < 0.05 from C6.9 CM and **P < 0.01 from C6.4 CM + anti-amphiregulin antibody). An isotype non specific antibody did not modify the response of C6.9 cells to C6.4 CM; the anti-amphiregulin antibody did not affect the viability of C6.9 cells incubated with C6.9 CM (data are omitted for clarity). (D) Effect of exogenous amphiregulin (100 ng/mL) and THC (2 μM) on C6.9 cell viability (n = 5; **P < 0.01 from vehicle-treated cells, **P < 0.01 from THC-treated cells, *P < 0.01 from amphiregulin-treated cells, **P < 0.01 from THC-treated cells). Veh: Vehicle, 5′NT: 5′ Nucleotidase, AMPHI: Amphiphysin 1, AREG: Amphiregulin, MGP: Matrix Gla protein, CD81: CD81 antigen, GAD1: Glutamate decarboxylase 1, SLC38A4: Amino acid transport system A3, OLIG1: Oligodendrocyte transcription factor 1, NR4A1: Nuclear receptor subfamily 4, group A, member 1, NRP1: Neuropilin1, EDNRA: Endothelin receptor type A and EMB: Embigin.
Amphiregulin Regulates the Activation of the ER Stress-Related THC-Induced Pro-Apoptotic Pathway

We have recently shown that THC induces apoptosis of tumor cells via up-regulation of the ER stress-related proteins p8 and TRB3 (Carracedo et al., 2006a,b). Therefore, we asked whether amphiregulin affects the sensitivity of tumor cells by regulating the activation of this pro-apoptotic route. Knock-down of amphiregulin levels or pharmacological inhibition of the ERK pathway in C6.4 cells increased the basal levels of p8 and TRB3 (Fig. 4A) and strongly enhanced the up-regulation of these genes in response to THC treatment (Fig. 4C). Likewise, addition of exogenous amphiregulin to C6.9 cells reduced the basal levels of p8 and TRB3 (Fig. 4B) and abrogated the induction of these genes in response to THC treatment (Fig. 4D). Taken together, these results indicate that amphiregulin—via ERK activation—promotes resistance to THC treatment via down-regulation of the genes involved in cannabinoid-induced cell death.

C6.4 Cell-Derived Tumors Are Resistant to Cannabinoid Antitumoral Action

In order to analyze the in vivo relevance of our findings, we generated tumors xenografts by injecting C6.4 and C6.9 cells subcutaneously in nude mice. Amphiregulin expression (Fig. 5A) and ERK phosphorylation (Fig. 5B, upper panels) were much higher in C6.4 than in C6.9 tumors. In addition, THC administration led to TRB3 up-regulation (Fig. 5B, middle panels), increased apoptosis (Fig. 5B, lower panels) and reduced tumor growth in C6.9 but not in C6.4 cell-derived tumors (Fig. 5C) supporting that increased expression of amphiregulin modulates the activation of the cannabinoid-induced pro-apoptotic pathway in vivo. In order to confirm these observations, we analyzed THC antitumoral action in C6.4 tumors in which amphiregulin expression had been knocked-down. As shown in Fig. 6, in vivo silencing of amphiregulin reduced the growth by itself and, more importantly, rendered C6-4 cell-derived tumors sensitive to THC treatment, demonstrating that amphiregulin expression is a factor of resistance to cannabinoid antitumoral action.

DISCUSSION

In this study we aimed to identify genes involved in the resistance of tumor cells to cannabinoid antitumoral action and found the heparin-bound EGF-like receptor ligand amphiregulin as a solid candidate. Pro-amphiregulin, the membrane-bound form of this protein, can be cleaved by selective proteases and released to the extracellular medium (Gschwind et al., 2003). Amphiregulin can subsequently bind EGFR family members and stimulate signaling routes, such as the ERK pathway, associated with cell survival and proliferation (Berasain et al., 2007; Gschwind et al., 2003; Schafer et al., 2004). Our evidence support the notion that increased amphiregulin expression by glioma cells mediates the resistance to cannabinoid-induced apoptosis through activation of the EGFR/ERK pathway: (i) amphiregulin levels and ERK phosphorylation are much higher in cannabinoid-resistant C6.4 cells than in cannabinoid-sensitive C6.9 cells, not only in culture but also in tumor xenografts; (ii) conditioned medium from C6.4 cells renders the C6.9 subclone resistant to THC-induced apoptosis in an amphiregulin-dependent fashion; (iii) knock-down of amphiregulin levels reduces EGFR and ERK phosphorylation and sensitizes C6.4 cells to cannabinoid treatment; (iv) exogenous amphiregulin enhances EGFR phosphorylation and promotes resistance to THC-induced cell death in an EGFR tyrosine kinase activity-dependent fashion; and (v) in vivo silencing of amphiregulin renders C6.4 tumors sensitive to THC antitumoral action.

Cannabinoid-induced apoptosis of tumor cells has been very recently shown to rely on the up-regulation of the stress-regulated protein p8 and several of its downstream targets including the pseudokinase TRB3 (Carracedo et al., 2006a,b). Interestingly, knock-down of amphiregulin levels or inhibition of the ERK pathway up-regulates basal p8 and TRB3 levels in the C6.4 subclone. These findings are in agreement with previous observations showing that activation of the ERK pathway inhibits p8 expression in pancreatic cancer cells (Malicet et al., 2003) and indicate that amphiregulin (via sustained ERK activation) regulates the expression of these genes in our model. In line with this idea, we found that tumors derived from the C6.9 sub-clone express higher levels of TRB3 than those derived from the C6.4 sub-clone. Likewise, amphiregulin levels and ERK phosphorylation are lower in C6.9 cell-derived tumors. Moreover, THC induces a much stronger up-regulation of the p8/TRB3 pro-apoptotic pathway in the cannabinoid-resistant C6.4 cells when amphiregulin levels have been knocked-down or the ERK pathway has been inhibited.
been inhibited. Similarly, TRB3 up-regulation and increased apoptosis upon THC treatment are observed in C6.9 but not in C6.4 cell-derived tumors. Taken together, these findings show that amphiregulin enhances the resistance of glioma cells to cannabinoid-induced apoptosis via ERK activation and subsequent down-regulation of the p8/TRB3 pathway.
We are aware that several G protein-coupled receptors, including the cannabinoid receptor CB1, have been shown to induce ERK stimulation through trans-activation of tyrosine kinase receptors (Gschwind et al., 2003; Hart et al., 2004). In particular, Ullrich’s group has shown that low doses of synthetic cannabinoids trigger amphiregulin cleavage and ERK activation in several types of tumor cells, an effect that was associated with increased cell proliferation (Hart et al., 2004). In this study we observed that the ratio P-ERK/total ERK is increased in response to amphiregulin stimulation.

Fig. 3. Increased amphiregulin expression induces ERK phosphorylation in C6 cells. (A) ERK phosphorylation status of unstimulated C6.9 and C6.4 cells (left panel), C6.4 cells transfected with siC or siAREG (middle panel), transfection efficiency was ≥85% and C6.9 cells incubated with C6.4 or C6.9 conditioned medium (CM) (right panel). Representative blots of 3 experiments are shown. Values represent the optical density relative to un-stimulated C6.9 cells (left panel), siC-transfected C6.4 cells (middle panel) or C6.9 cells incubated with C6.9 CM (right panel) [mean ± S.D.; n = 3; ** P < 0.01 from un-stimulated C6.9 cells (left panel), siC-transfected C6.4 cells (middle panel) or C6.9 cells incubated with C6.9 CM (right panel)]. (B) Effect of THC and U0126 (5 μM) on the viability of C6.4 cells (n = 5; ** P < 0.01 * P < 0.05 from vehicle-treated cells; THC 2.5 μM for C6.4 cell treatment). (C) Effect of THC and U0126 (5 μM) on the viability of C6.9 cells (n = 5; ** P < 0.01 from vehicle-treated cells; THC 1.75 μM for C6.9 cell treatment). (D) Pre-incubation with U0126 (5 μM) prevents the protective effect of exogenous AREG on THC-induced C6.9 cell death (n = 4; ** P < 0.01 from vehicle-treated cells, ** P < 0.01 from THC-treated cells, ** P < 0.01 from THC+AREG-treated cells; THC 1.75 μM for C6.9 cell treatment).
consistently higher in C6.4 cells even when non-stimulated C6.4 cells are compared with THC-stimulated C6.9 cells. We therefore believe that, at least in this model, the basal tone of ERK phosphorylation rather than the ability of cannabinoids to stimulate this route is the important factor that determines the resistance of tumor cells to THC-induced apoptosis.

GBM exhibits a high resistance to standard chemotherapy and radiotherapy (Maher et al., 2001; Nieder et al., 2006; Wong et al., 2007). Identification of the molecular markers associated to the resistance of glioma cells to each type of therapeutic agent, in combination with a better understanding of the alterations that drive the high rate of proliferation and invasiveness of these tumors, could lead to the establishment of individualized targeted therapies capable of reducing the extreme malignancy of these tumors. During the last few years, work performed by our group and others has shown that cannabinoids induce apoptosis of glioma cells and reduce tumor growth in animal models of gliomas [reviewed in (Guzman, 2003; Velasco et al., 2004)]. Moreover, a pilot clinical trial has recently tested the antitumoral activity of THC in recurrent glioblastoma multiforme. This study proved the safety of THC administration and pointed to a decreased proliferation (Guzman et al., 2006) and an increased apoptosis (Guzman et al., 2006) of tumor cells in THC-treated patients. The study also suggested that THC produces a modest beneficial effect on median patient survival, similar to that obtained in clinical trials for recurrent glioblastomas with temozolamide, the current benchmark for the management of these tumors (Guzman et al., 2006). Results presented here show that increased amphiregulin expression is a factor associated with the resistance of glioma cells to cannabinoid treat-
Fig. 5. THC induces TRB3 up-regulation and apoptosis in C6.9 but not in C6.4 cell-derived tumors. (A) Amphiregulin mRNA levels in C6.9 and C6.4 cell-derived tumor xenografts as determined by real-time quantitative PCR (n = 4 for each condition; **P < 0.01 from vehicle-treated C6.9 tumors). (B) Phospho-ERK1/2 (upper panel) or TRB3 (middle panel) immunostaining of C6.9 (left) and C6.4 (right) cell tumor xenografts. Values inside the photomicrographs are expressed as the phospho-ERK1/2 or TRB3-stained area relative to the number of nuclei in each field and correspond to 10 fields of 3 different tumors for each condition. Data are normalized using vehicle-treated C6.9 tumors (for phospho-ERK1/2 levels) and C6.4 vehicle-treated tumors (for TRB3 levels) as a reference. Representative photomicrographs are shown (***P < 0.01 from vehicle-treated tumors). Lower panel: Effect of THC on apoptosis of C6.9 (left) and C6.4 (right) tumor xenografts. Values inside the photomicrographs are expressed as the percentage of TUNEL-positive cells relative to the total number of nuclei in each field and correspond to 10 representative fields of 3 different tumors for each condition. Representative photomicrographs are shown (***P < 0.01 from vehicle-treated tumors). (C) Effect of THC on the growth of tumor xenografts derived from C6.9 (left panel) and C6.4 (right panel) cells in nude mice (n = 8 for each condition; **P < 0.01 and *P < 0.05 from vehicle-treated tumors).
ment and suggest that the combined administration of cannabinoids and inhibitors of the EGFR signaling pathway and/or siRNAs to selectively reduced the expression of resistance factors (such as amphiregulin) may be a therapeutic strategy to improve the antitumoral activity of cannabinoid-based therapies.

ACKNOWLEDGMENTS

M.L. was recipient of a Juan de la Cierva contract from the Spanish Ministry of Education and Science. A.C. was recipient of a fellowship from Consejería de Educación del Gobierno Vasco. S.T. was recipient of a

GLIA
research contract from Consejería de Educación de la Comunidad Autónoma de Madrid. S.H.-T. has a technician contract from Spanish Ministry of Education and Fondo Social Europeo. M.S. was recipient of a fellowship from MEC.

REFERENCES


AQ1: Please provide all author names for all “et al.-type” references, as required by journal style.

AQ2: Please check whether the grant information is OK as typeset.


AQ2: This is OK