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Cannabinoid Receptor Activation Induces Apoptosis through Tumor Necrosis Factor α – Mediated Ceramide *De novo* Synthesis in Colon Cancer Cells

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Abstract Purpose: Cannabinoids have been recently proposed as a new family of potential antitumor agents. The present study was undertaken to investigate the expression of the two cannabinoid receptors, CB₁ and CB₂, in colorectal cancer and to provide new insight into the molecular pathways underlying the apoptotic activity induced by their activation.

Experimental Design: Cannabinoid receptor expression was investigated in both human cancer specimens and in the DLD-1 and HT29 colon cancer cell lines. The effects of the CB₁ agonist arachinodol-2'-chloroethylamide and the CB₂ agonist *N*-cyclopentyl-7-methyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (CB13) on tumor cell apoptosis and ceramide and tumor necrosis factor (TNF)- α production were evaluated. The knockdown of TNF- α mRNA was obtained with the use of selective small interfering RNA.

Results: We show that the CB₁ receptor was mainly expressed in human normal colonic epithelium whereas tumor tissue was strongly positive for the CB₂ receptor. The activation of the CB₁ and, more efficiently, of the CB₂ receptors induced apoptosis and increased ceramide levels in the DLD-1 and HT29 cells. Apoptosis was prevented by the pharmacologic inhibition of ceramide *de novo* synthesis. The CB₂ agonist CB13 also reduced the growth of DLD-1 cells in a mouse model of colon cancer. The knockdown of TNF- α mRNA abrogated the ceramide increase and, therefore, the apoptotic effect induced by cannabinoid receptor activation.

Conclusions: The present study shows that either CB₁ or CB₂ receptor activation induces apoptosis through ceramide *de novo* synthesis in colon cancer cells. Our data unveiled, for the first time, that TNF- α acts as a link between cannabinoid receptor activation and ceramide production.

Cannabinoids, the active components of *Cannabis sativa* and their derivatives, exert a wide spectrum of central and peripheral actions through the activation of specific receptors that are normally bound by a family of endogenous ligands, the endocannabinoid anandamide and 2-arachidonoylglycerol (1, 2). Two cannabinoid receptors have been characterized and

cloned thus far: CB₁ (3) and CB₂ (4). CB₁ receptors are particularly abundant in discrete areas of the brain, where they mediate cannabinoid psychoactivity, and are also expressed in peripheral nerve terminals and various extraneural sites, such as the testis, uterus, eye, and spleen (1–4). In contrast, CB₂ receptors are believed to be almost exclusively expressed in the cells and organs of the immune system and are unrelated to cannabinoid psychoactivity (4). Nevertheless, they have also been recently shown in cells from other origins, especially tumor cells (5, 6).

Experimental evidence has shown that cannabinoid administration can inhibit the growth of several models of tumor xenografts in rats and mice (7–11). This antitumor action of cannabinoids relies on the ability of these drugs to inhibit tumor angiogenesis (12) or directly induce apoptosis or cell cycle arrest in neoplastic cells (7–11). Cannabinoid receptors have been shown to modulate several signaling pathways involved in the control of cell survival, including extracellular signal-regulated kinase (ERK; ref. 13), c-Jun-NH2-kinase (14), p38 mitogen-activated protein kinase (MAPK; ref. 15), and the ceramide pathway (16, 17). These findings point to the potential application of cannabinoids as antitumor agents (5, 6). However, the majority of these studies have been done with the use of psychoactive cannabinoids, in particular δ^9 -tetrahydrocannabinol, the main active component of marijuana,

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Translational Relevance

The present study shows that the antitumor actions of cannabinoid receptor agonists on colon cancer cells may be exerted either via the CB₁ receptor or, more efficiently, via the CB₂ receptor. The fact that selective targeting of CB₂ receptor results in colorectal tumor growth inhibition is of potential clinical interest for future cannabinoid-based anticancer therapies because the use of CB₂-selective ligands is not linked to the typical marijuana-like psychoactive effects of CB₁ activation. Moreover, we showed that only the CB₂ receptor is expressed by tumor cells in colorectal cancer human specimens and, thus, it is likely that only compounds with high selectivity for this receptor may be effective as anticancer agents in humans. The recent synthesis of new, highly selective CB₂ agonists opens the very attractive clinical possibility of using these compounds as adjuvants to conventional chemotherapeutic regimens for the treatment of colorectal cancer.

and WIN, 55,212-2, a nonselective synthetic cannabinoid agonist. It would be preferable, instead, to have cannabinoid-based therapeutic strategies that are devoid of typical CB₁ receptor-mediated psychotropic side effects. Hence, the recent synthesis of new, highly selective CB₂ agonists (18) opens the very attractive clinical possibility of targeting this receptor selectively.

Ceramide is a ubiquitous sphingolipid messenger that plays an important role in the control of tumor cell fate (19). CB₁ receptor activation has been shown to induce sphingomyelin hydrolysis and acute ceramide production within minutes in both primary astrocytes (20) and C6 glioma cells (16). The functional coupling of CB₁ receptors to sphingomyelinases might involve different adaptor proteins, one of which is the factor associated with neutral sphingomyelinase activation (FAN). FAN binds to a cytoplasmatic nine-amino-acid motif of the 55-kDa tumor necrosis factor (TNF) receptor, the neutral sphingomyelinase-activating domain, thereby coupling the receptor to sphingomyelin breakdown (21). On the contrary, sustained ceramide accumulation through enhanced *de novo* synthesis seems to play a major role in CB₂ receptor activation-induced apoptosis in tumor cells, including glioma (8, 16, 22), leukemia (23), and pancreatic cancer (9) cells. However, the precise molecular mechanisms involved in the CB₂ receptor-mediated generation of ceramide are still unknown.

TNF- α is one of the most pleiotropic cytokines acting as a host defense factor in a number of immunologic responses and antitumor activity (24, 25). The deregulation of TNF- α signaling results in a wide spectrum of human diseases, including sepsis, multiple sclerosis, rheumatoid arthritis, and cancer (26). It has been shown that TNF- α exerts a cytostatic or cytotoxic effect in several types of cells, and this effect is mediated, at least in part, by the stimulated production of ceramide (27–29). A large body of evidence has shown that the cannabinoid system is involved in the regulation of the cytokine network (30). However, the effects of cannabinoids on cytokines and, in particular, TNF- α production are

often conflicting and can be either stimulatory or inhibitory, depending on the experimental model system and the type of cell investigated. To our knowledge, the influence of cannabinoid receptor activation on TNF- α production in cancer cells has not yet been addressed.

Although it is well known that the endogenous cannabinoid system and cannabinoid receptors regulate gastrointestinal functions, such as gastric emptying, secretion, and intestinal motility (31, 32), few studies have investigated the expression and role of CB₁ and/or CB₂ receptors in normal (33) or neoplastic (15, 34) epithelial cells of the colon in humans. The reported results have shown that both inhibition of proliferation (34) and induction of apoptosis (15) in colon cancer cells are mediated by CB₁ receptor activation whereas little or no activity has been observed for the CB₂ receptor.

The aim of this study was to investigate whether even CB₂ receptor activation is involved in the antitumor action of cannabinoids in colon cancer cells and to identify the possible molecular mechanisms underlying this effect. In particular, we explored the hypothesis that cannabinoid receptor activation might induce a proapoptotic effect through a TNF- α -mediated increase in ceramide production.

Materials and Methods

Cell culture and drugs. DLD-1, HT29, LoVo, HCT8, SW480, HCA7, and HCT15 colon cancer cells were purchased from Interlab Cell Line Collection. The cells were cultured as previously described (35). The CB₁ receptor agonist arachinodol-2'-chloroethylamide (ACEA), the CB₁ antagonist AM251, and the CB₂ antagonist AM630 were purchased from Tocris Bioscience. The newly synthesized CB₂ agonist *N*-cyclopentyl-7-methyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1H)-on-3-carboxamide (CB13) was supplied by C.M. (18). The ceramide synthase inhibitor fumonisins B1 was purchased from Cayman Chemical Co.

Human tumor samples. Tissue samples were obtained from 24 patients (18 males, 6 females; median age: 68.5 y; age range: 58–77 y) who had undergone surgical resections for primary sporadic colorectal adenocarcinoma. All patients were thoroughly informed about the aims of the study and gave their written consent for the investigation in accordance with the ethical guidelines of our University. The tumor distribution was as follows: 10 (41.6%) in the proximal colon, 7 (29.2%) in the distal colon, and 7 (29.2%) in the rectum. The tumors were classified into four stages according to the American Joint Committee on Cancer staging system (36): stage I (T₁–T₂, N₀, M₀; n = 3), stage II (T₃–T₄, N₀, M₀; n = 14), stage III (any T, N_{1–2}, M₀; n = 4), and stage IV (any T, any N, M₁; n = 3).

Cancer tissue (from the edge of the tumor) and adjacent normal mucosa (at least 10 cm from the tumor) were excised from each surgical specimen. The samples were flash frozen in liquid nitrogen for PCR analysis and frozen at -80°C for Western blot analysis. Other samples were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis.

Western blot analysis. The total proteins from the tissue samples were obtained as described previously (37). DLD-1, HT29, and LoVo cells were grown to subconfluence and starved for 24 h in 0.1% FCS-supplemented media. After incubation in the absence or presence of drugs, the cells were washed in PBS and lysed with radioimmunoprecipitation assay buffer [0.9% NaCl, 20 mmol/L Tris-HCl (pH 7.6), 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.01% leupeptin]. The total proteins (70 μ g) as evaluated with the use of a bicinchoninic acid protein assay from tissue or cultured cells were subjected to Western blotting and immunoblotting analysis as previously described (35). The loading

and transfer of equal amounts of proteins were ascertained by either reblotting the membrane with an antitubulin antibody or staining the membrane with Ponceau S. The primary antibodies used were anti-CB₁ and anti-CB₂ rabbit polyclonal antibody (1:250; Alexis Biochemicals), anti-caspase-3 (1:500; Santa Cruz Biotechnology, Inc.), and antitubulin goat polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Inc.). The binding of each primary antibody was determined by the addition of suitable peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit antibodies 1:5,000, and anti-goat antibody 1:10,000; Amersham). Human spleen tissue served as CB₁ and CB₂ positive control. Densitometric analysis was done with the ImageJ software.

Reverse-transcriptase PCR. The total RNA was extracted from the tissue samples and cells with the use of the RNeasy kit (Qiagen) according to the manufacturer's instructions. One microgram of total

RNA was retrotranscribed to cDNA with the Immoprom-II Reverse Transcriptase kit (Promega) and amplified with the use of the GoTaq (Promega) according to the manufacturers' instructions. Cannabinoid receptor mRNA was detected with the QuantiTect Primer Assay (QT00998823 and QT00012376 for CB₁ and CB₂ receptor, respectively; Qiagen) in nonquantitative assays. For glyceraldehydes-3-phosphate dehydrogenase (GAPDH), the primer sequences were 5'-ACCACCATG-GAGAAGGCTGG-3' (forward) and 5'AAGTTGTCGCTGTGGGTG-3' (reverse). The size of CB₁, CB₂, and GAPDH reverse transcription-PCR products was 148, 87, and 196 bp, respectively.

Immunofluorescence analysis. The DLD-1 and HT29 cells (2×10^5) were seeded onto glass coverslips (15×15 mm). After 24 h, the cells were washed twice with 1 mL of cold PBS and fixed for 20 min in 3.7% paraformaldehyde in PBS. After three washes for 2 min with PBS, the cells were permeabilized with 1 mL 0.25% Triton X-100 in PBS for

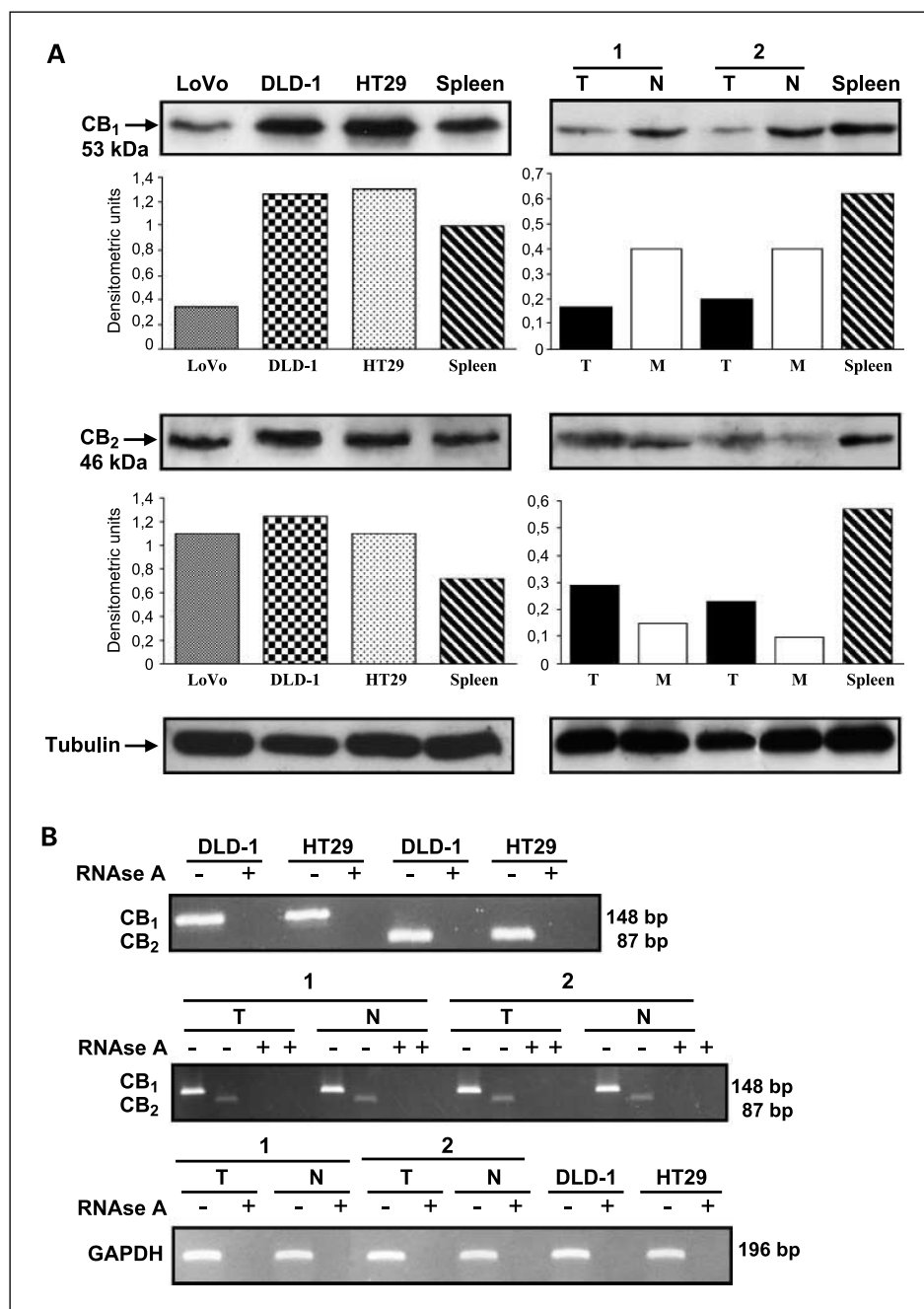


Fig. 1. Expression of cannabinoid receptors in colon cancer cells and human specimens. *A*, Western blot analysis. Expression of CB₁ and CB₂ receptor in the LoVo, DLD-1, and HT29 colon cancer cells, and in representative paired adenocarcinoma and adjacent normal mucosa from two patients. The samples were normalized for protein loading (70 μ g) by reblotting the membrane-bound protein with an antitubulin antibody. The densitometric histograms of relative band intensities are shown. Human spleen was used as a positive control for CB₁ and CB₂ receptor expression. N, normal mucosa; T, tumor; kDa, kilodaltons. *B*, qualitative reverse transcription-PCR. Expression of CB₁ and CB₂ receptor mRNA in the DLD-1 and HT29 colon cancer cells (*top*), and in representative paired adenocarcinoma and adjacent normal mucosa from two patients (*middle*). The housekeeping gene GAPDH was used as an internal control (*bottom*). RNase-treated samples were used as negative controls. N, normal mucosa; T, tumor.

5 min at room temperature and washed thrice for 5 min in PBS. All the following treatments were done in the dark. After the staining of nuclei with Hoechst 33258 (blue fluorescence; Sigma-Aldrich) diluted 1:1000 in PBS for 30 min at 37°C, the cells were washed thrice for 5 min with 1 mL PBS at room temperature and incubated in 1 mL of blocking buffer (3% bovine serum albumin, 0.1% Triton X-100 in PBS) for 1 h at room temperature and then incubated with the primary rabbit CB₁ or CB₂ polyclonal antibody (Cayman Chemical Co.) diluted 1:200 in blocking buffer overnight at 4°C. The next day, the cells were washed thrice for 15 min each in washing buffer (0.1% Triton X-100 in PBS) and incubated with the secondary Rodamine-conjugated anti-rabbit antibody (red fluorescence; Santa Cruz Biotechnology, Inc.) diluted 1:800 for 60 min at room temperature, washed thrice with 1 mL of washing buffer for 5 min at room temperature, dried, mounted onto glass slides, and examined with a Nikon Eclipse TE2000-U (Nikon J.P.) confocal microscopy. Confocal images (1024 × 768 pixels) were obtained with the use of an objective lens (magnification, ×63).

Immunohistochemistry. Four-micrometer-thick sections were cut from formalin-fixed and paraffin-embedded tissue blocks and processed as described previously (37). For CB₁/CB₂ receptor detection, the sections were incubated in 10 mmol/L citrate buffer (pH 6) in a microwave oven for 5 min. The sections were blocked in 5% bovine serum albumin in TBS (pH 9) for 1 h before the application of primary antibodies. CB₁ and CB₂ antibodies (Cayman Chemical Co) at 1:200 dilution in TBS (pH 9) were incubated overnight at 4°C. The slices were then washed again in PBS and incubated with secondary antibody (rabbit anti-goat IgG horseradish peroxidase conjugated; Zymed Laboratories) for 25 min. For ceramide detection, the slices were washed in PBS (0.1 mol/L; pH 7.4) and then incubated with the primary antibody (MID 15B4; Sigma-Aldrich) at 1:10 concentration for 18 h overnight at 4°C. The slices were then washed again in PBS and incubated with secondary antibody (anti-mouse IgG peroxidase conjugate 1:300; Sigma-Aldrich) for 30 min. In all cases, the detection of the antibody complex was done with the use of 3,3'-diaminobenzidine tetrahydrochloride-plus kit substrate for horseradish peroxidase (Zymed Laboratories). As a negative control for CB₁, CB₂, and ceramide staining, the tissue sections were treated with normal serum instead of each primary antibody.

The extent of CB₁ and CB₂ staining in tumor samples was recorded through a three-grade system based on the percentage of tumor epithelial cells stained: grade 0 = 1% to 20%, grade 1 = 21% to 70%, and grade 2 = more than 70%. The ceramide staining in tumor xenografts was graded on the basis of the intensity of positive cells: none/weak and moderate/strong.

Caspase-3 activity determination. The activity of caspase-3 was determined with the use of a fluorescent substrate according to the method previously described (37). The determinations were done in quintuplicate. The data were expressed as arbitrary units per milligram of proteins.

Flow cytometric analysis. The cells were seeded at 5×10^5 per well in six-well plates and treated with test drugs. For the determination of apoptosis, they were washed in Annexin binding buffer containing 125 mmol/L NaCl, 10 mmol/L HEPES/NaOH (pH 7.4), and 5 mmol/L CaCl₂. The cells were then stained with the combination of Annexin V FITC and 7-amino-actinomycin D (7-AAD; Beckman Coulter, Inc.). The samples were measured by flow cytometric analysis on a Coulter XL flow cytometer (Coulter XL; Beckman Coulter) with the use of the EXPO 32ADC Software (Beckman Coulter). The determinations were done in triplicate, and the data on apoptotic cells were expressed as percentage of total cells counted.

The ceramide content was determined according to the method previously described (37). The determinations were done in triplicate, and the data were expressed as mean fluorescence intensity of positive stained cells.

Cytotoxic assay. The sulforhodamine-B protein staining assay (Sigma-Aldrich) was used for the measurement of *in vitro* cytotoxicity. The cells were seeded in 96-microwell plates (10^5 cells in 200 μ L for

each well). After 24-h preincubation, increasing concentrations of CB13 were added to the plates, which were incubated for another 48 h. As the end point measurement, the sulforhodamine-B test was done according to the procedure described by Skehan et al. (38). Briefly, the cells were fixed with 80% trichloroacetic acid for 1 h at 4°C and then washed five times with distilled water. The trichloroacetic acid-fixed cells were stained for 30 min with 0.4% sulforhodamine B dissolved in 1% acetic acid. At the end of the staining period, the sulforhodamine B was removed, and the wells were rinsed four times with 1% acetic acid. Bound dye was dissolved with 10 mmol/L unbuffered Tris base (pH 10.5) for 5 min on a shaker. The absorbance was measured at 564 nm with a multilabel plate counter (Wallace Victor2; Perkin-Elmer). The determinations were done in triplicate, and the data were expressed as percentage cell survival compared with vehicle treatment regarded as 100%.

Tumor xenografts. Tumors were induced by s.c. flank injection of 2×10^6 DLD-1 or HT29 cells in PBS supplemented with 0.1% glucose in immunodeficient nude (BALB/c) mice. When the tumors reached

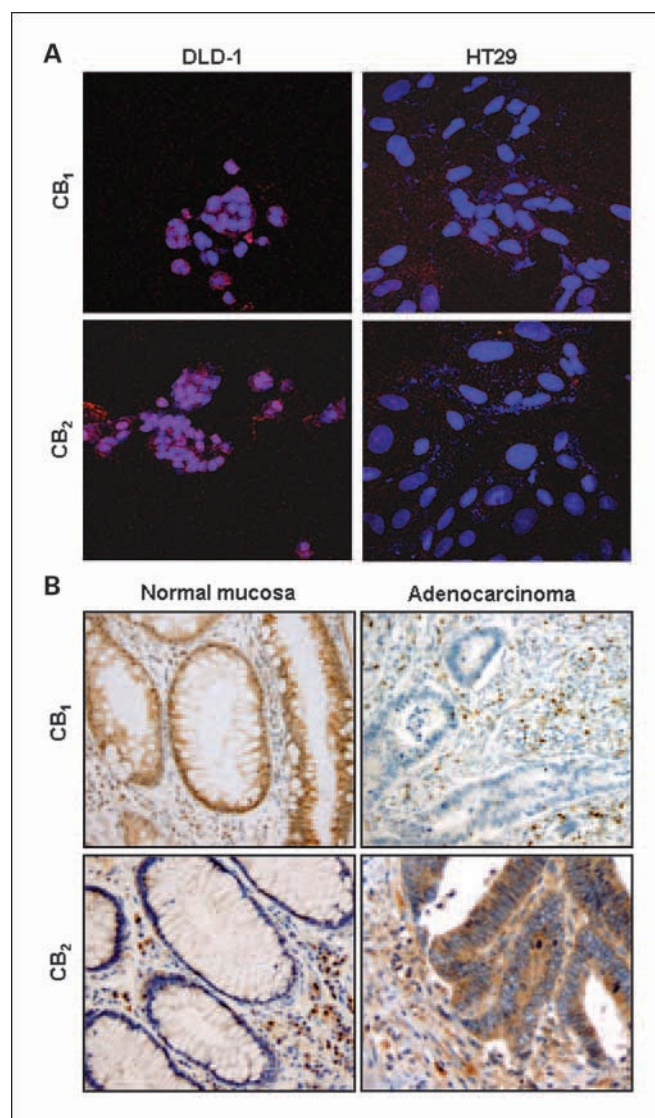
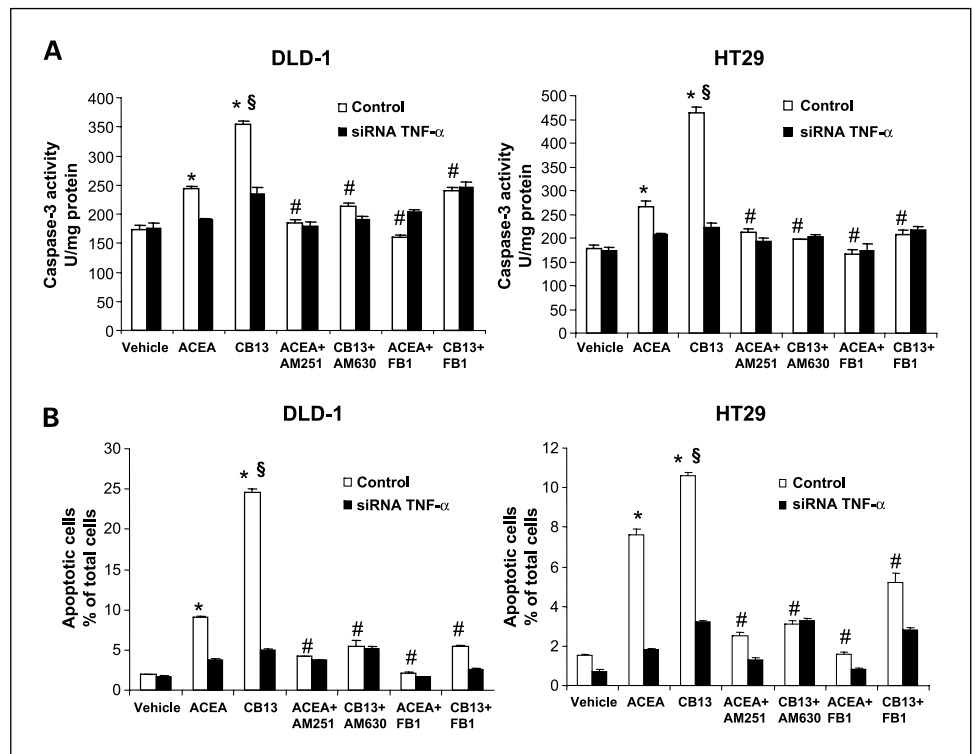


Fig. 2. Expression of cannabinoid receptors in colon cancer cells and human specimens. *A*, immunofluorescence analysis of CB₁ and CB₂ receptor expression in the DLD-1 and HT29 colon cancer cells. Representative (×63) images of cannabinoid receptor-stained colon cancer cells. Red, cannabinoid receptors; blue, nuclei. *B*, immunohistochemical analysis of CB₁ and CB₂ receptors in two representative paired human cancer specimens and adjacent normal colon mucosa. Hematoxylin counterstain. Original magnification, ×200.

Fig. 3. Effects of 100 nmol/L ACEA, 100 nmol/L CB13, ACEA + 100 nmol/L AM251, CB13 + 100 nmol/L AM630, ACEA + 10 μ mol/L FB1 and CB13 + FB1 on caspase-3 activity (A) and cell apoptosis (B) in the DLD-1 and HT29 colon cancer cells transfected with either control or TNF- α – selective siRNA (siRNA TNF- α). Columns, mean of three different experiments; bars, SE. *, significant increase compared with vehicle treatment ($P < 0.05$); #, significant decrease compared with treatment with ACEA or CB13 ($P < 0.05$); §, significantly different compared with treatment with ACEA ($P < 0.05$). FB1, fumonisin B1.



an average size of 100 mm³, the animals were assigned randomly to one of the two groups ($n = 8$ for each group) and injected peritumorally for 12 d with CB13 (2.5 mg/kg/d) or vehicle in 100 μ L of PBS supplemented with 5 mg/mL defatted and dialyzed bovine serum albumin. The tumors were measured with an external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

TNF- α measurement. The levels of TNF- α in the DLD-1 and HT29 cells, and in the tumor xenografts were measured with the use of a commercial ELISA kit (Cayman Chemical Co.) according to the protocol provided by the manufacturer. The determinations were done in quadruplicate and were expressed as picograms per microgram of protein.

RNA interference and transfections. DLD-1 and HT29 cells (7×10^5) were seeded onto 6-well plates and, after 24 h, treated with either a small interfering RNA (siRNA; Qiagen) directed against the TNF- α or a siRNA (Qiagen) directed against the GFP gene as a control (40 nmol/L). The TNF- α sequences targeted by siRNA are siRNA 1: 5'-AACCCAAGCT-TAGAACTTAA-3' and siRNA 2: 5'-CCGACTCAGCGCTGAGATCAA-3'. The GFP sequence targeted by the siRNA is siRNA 3: 5'-CGGCAAGCT-GACCCGTAAGTTCAT-3'. Forty-eight hours post siRNA transfection, the supernatant was collected to analyze the basal TNF- α levels, or the cells were treated with test drugs.

Statistical analysis. The caspase-3 activity, flow cytometric determination of cell apoptosis and ceramide synthesis, percentage cell survival, TNF- α production, and tumor volumes in mice were expressed as mean \pm SE. The differences in these parameters were compared through the paired-value Wilcoxon test or the Mann-Whitney test as appropriate. The differences in ceramide immunostaining intensity were compared with the use of the χ^2 test. Statistical analysis was done with the Stata Statistic software (release 5.0; Stata Corp.). All the P resulted from the use of two-sided statistical tests; a $P < 0.05$ was considered statistically significant.

Results

Colorectal cancer cells express cannabinoid receptors. First, we determined the expression of cannabinoid receptors in the

DLD-1, HT29, and LoVo human colon cancer cells by Western blot analysis. CB₁ and CB₂ cannabinoid receptor proteins were expressed in all three cell lines (Fig. 1A). Because the HT29 and DLD-1 cells showed, respectively, the highest levels of either CB₁ or CB₂ cannabinoid receptor protein expression (Fig. 1A), subsequent experiments were done on these two cell lines. Reverse transcription-PCR showed detectable levels of mRNA for both CB₁ and CB₂ receptors in the same cells (Fig. 1B) whereas immunofluorescence analysis confirmed their expression at a protein level (Fig. 2A). CB₁ and CB₂ cannabinoid receptor protein and mRNA were also expressed in both human tumor and normal mucosa specimens (Fig. 1A and B). However, Western blot experiments showed a higher amount of CB₁ receptor protein in the normal mucosa than in the tumor tissue in 20 paired specimens (Fig. 1A). In 4 patients, very low levels of CB₁ protein were found in both normal and neoplastic specimens. Unlike CB₁, the expression of CB₂ had increased in 22 of the 24 tumor specimens when compared with paired normal mucosa (Fig. 1A). No CB₂ protein expression was noted in two paired normal mucosa and tumor specimens. Immunohistochemistry confirmed this different epithelial expression profile of the two cannabinoid receptors; CB₁ receptor was mainly expressed by absorptive crypt epithelium in normal mucosa whereas its immunoreactivity was very slight in neoplastic epithelial cells: 19 tumors (79.1%) were grade 0, 4 (16.7%) were grade 1, and 1 (4.2%) was grade 2 (Fig. 2B). On the contrary, CB₂ staining was weak in normal colon epithelium whereas it was intense in colorectal cancer cells: 5 tumors (20.8%) were grade 0, 12 (50.0%) were grade 1, and 7 (29.2%) were grade 2 (Fig. 2B). CB₁ and CB₂ positive staining was also found, respectively, in the subepithelial smooth muscle cells and the subepithelial interstitial cells, most likely macrophages (Fig. 2B). The extent of CB₁ and CB₂

receptor expression in the tumor samples did not significantly vary with respect to the tumor site or stage.

Cannabinoid receptor agonists induce apoptosis in colon cancer cells. We tested the functionality of cannabinoid receptors in the control of colon cancer cell growth by using the synthetic cannabinoid CB₁ agonist ACEA and the CB₂ agonist CB13. We first evaluated whether ACEA and CB13 were involved in inducing the early events of the apoptotic process (i.e., caspase-3 activation). The treatment of both DLD-1 and HT29 cells with 100 nmol/L ACEA and CB13 resulted in a decrease in procaspase-3 levels (Supplementary Fig. S1) and, as a consequence, in a significant increase in caspase-3 activation (Fig. 3A). Flow cytometric detection of cell apoptosis confirmed these findings; the treatment of both DLD-1 and HT29 cells with either ACEA or CB13 produced a significant increase in the number of apoptotic cells when compared with vehicle treatment (Fig. 3B). In both experiments, CB13 administration was significantly more efficient than that of ACEA (Fig. 3A and B). ACEA-mediated and CB13-mediated apoptosis were prevented by the administration of the CB₁ antagonist AM251 or the CB₂ antagonist AM630, respectively, thus indicating the receptor specificity of the two types of response (Fig. 3A and B).

Cytotoxic effect of CB₂ agonist in colon cancer cells. To investigate whether CB13 could have a growth inhibitory effect on other colon cancer cells, HCT8, SW480, HCA7, and HCT15 cells were treated with increasing concentrations of the compound. The four cell lines have been shown to express the CB₂ receptor in a previously published study (15). The sulforhodamine-B protein staining assay was used to determine cytotoxicity. The results show that the growth of all cell lines was inhibited in a dose-dependent manner. CB13 had similar cytotoxic effects against the cell lines tested with significant loss of viability at concentrations >50 nmol/L (Supplementary Fig. S2).

Antitumor effect of CB₂ agonist in colon cancer models in vivo. To confirm the novel findings of CB₂-mediated proapoptotic effects *in vitro*, we investigated the antitumor activity of CB13 *in vivo*. We first generated tumor xenografts by s.c. injection of either DLD-1 or HT29 cells in immunodeficient mice. Western blot analysis showed that tumors obtained with DLD-1 cells showed a higher expression of CB₂ receptor than those obtained with HT29 (Fig. 4A). Therefore, we tested the tumor growth inhibitory effect of CB13 on DLD-1 colon cancer models. As shown in Fig. 4B and C, peritumoral treatment with CB13 significantly reduced the growth of the established colon tumors.

De novo synthesized ceramide mediates the proapoptotic effect induced by cannabinoid receptor activation. As shown in Fig. 5A and Supplementary Fig. S3A and B, incubation with ACEA or CB13 led to ceramide accumulation in the DLD-1 and HT29 cells, and this effect was prevented by the administration of the ceramide synthase inhibitor fumonisin B1. The ceramide increase was significantly higher after CB13 treatment than ACEA treatment. Moreover, treatment with fumonisin B1 prevented the ACEA/CB13-mediated procaspase-3 decrease (Supplementary Fig. S1), activation of caspase-3 (Fig. 3A), and induction of apoptosis (Fig. 3B). We also investigated the immunohistochemical expression of ceramide in tumors generated in mice. The administration of CB13 increased ceramide expression in tumor epithelial cells when compared with treatment with only vehicle (Fig. 5B). Altogether these

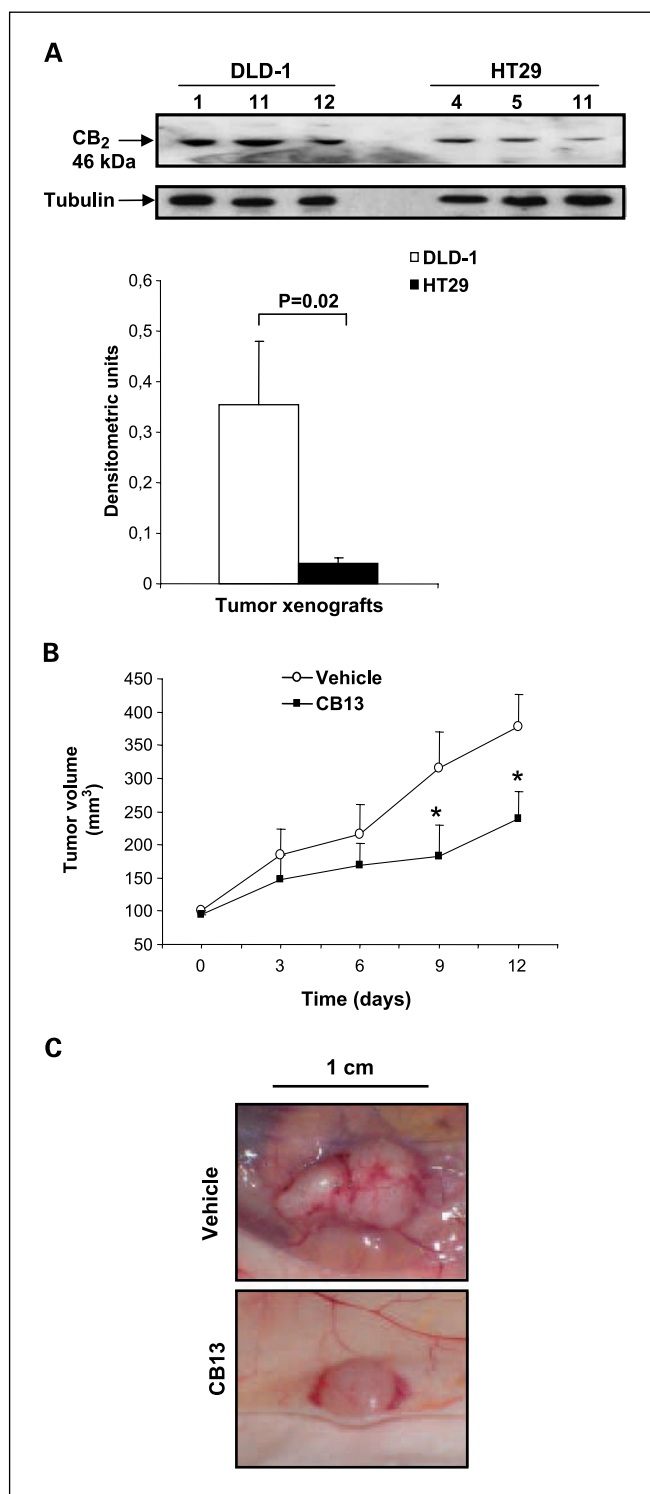
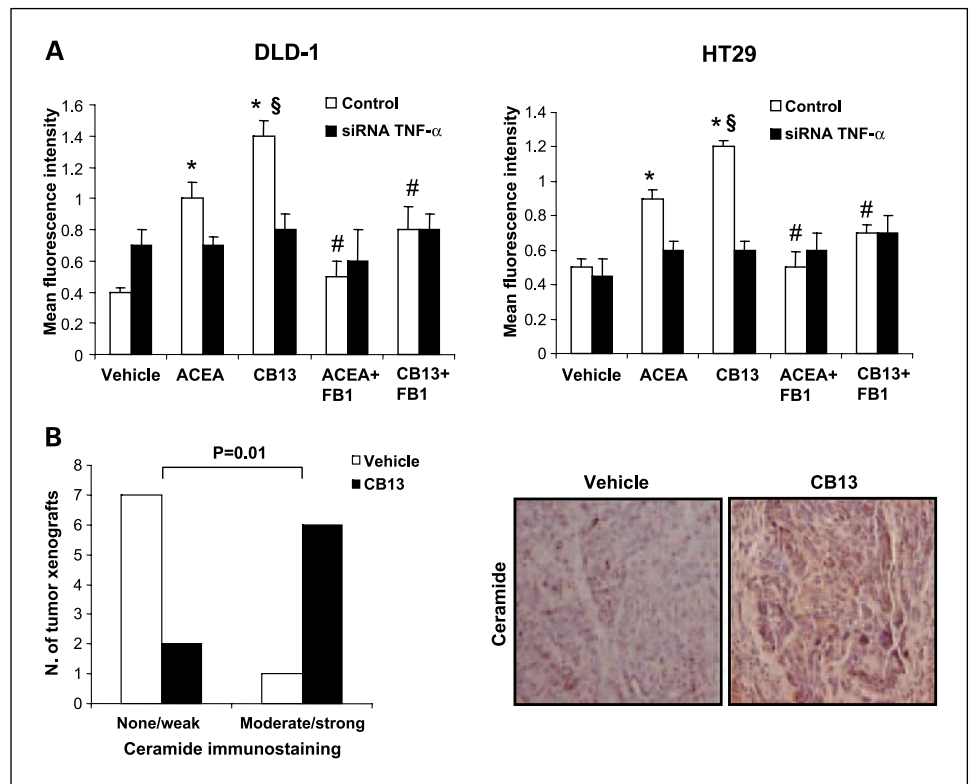


Fig. 4. *In vivo* antitumor activity of CB13. **A**, Western blot analysis. Expression of CB₂ receptor in tumors generated by s.c. injection of either DLD-1 (tumors 1, 11, and 12) or HT29 (tumors 4, 5, and 11) colon cancer cells in BALB/c mice. The samples were normalized for protein loading (70 μ g) by reblotting the membrane-bound protein with an antitubulin antibody. The densitometric histograms of relative band intensities are shown. Columns, mean of three different experiments; bars, SE. **B**, tumors generated by s.c. injection of DLD-1 cells in BALB/c mice. The animals were treated with either vehicle or CB13 (2.5 mg/kg/d) for up to 12 d ($n = 8$ for each experimental group). The tumor volume was monitored during the treatment. *, significantly different from vehicle-treated tumors at the corresponding day of treatment ($P < 0.05$). **C**, photographs of a representative vehicle-treated and CB13-treated tumor.

Fig. 5. Ceramide production. **A**, variations in ceramide production as determined by flow cytometry, after treatment with 100 nmol/L ACEA, 100 nmol/L CB13, ACEA + 10 μ mol/L FB1 and CB13 + FB1 in the DLD-1 and HT29 cells transfected with either control or TNF- α -selective siRNA (siRNA TNF- α). Columns, mean of three determinations of ceramide-positive cells given in mean fluorescence intensity of stained cells; bars, SE. *, significant increase compared with vehicle treatment ($P < 0.05$); #, significant decrease compared with ACEA or CB13 treatment ($P < 0.05$); §, significantly different compared with treatment with ACEA ($P < 0.05$). **B**, ceramide immunostaining intensity in tumors generated by s.c. injection of DLD-1 cells in BALB/c mice after treatment with either vehicle or CB13 (left) and representative images of ceramide immunostaining in tumor xenografts treated with either vehicle (none/weak intensity) or CB13 (moderate/strong intensity; right). Hematoxylin counterstain. Original magnification, $\times 200$.



findings indicate that the *de novo* synthesized ceramide is involved in CB₁/CB₂ receptor-induced apoptosis in colon cancer cells.

Cannabinoid receptor activation stimulates TNF- α production. The administration of either 100 nmol/L ACEA or CB13 determined a significant increase in TNF- α synthesis in both DLD-1 and HT29 cells (Fig. 6A). The TNF- α induction was maximal after 48 hours of treatment with the agonists. The TNF- α levels were significantly higher after treatment with CB13 than with ACEA (Fig. 6A). The administration of either the CB₁ antagonist AM251 or the CB₂ antagonist AM630 prevented the TNF- α increase induced by ACEA or CB13, respectively (Fig. 6A). In tumor xenografts, the TNF- α levels were significantly higher in neoplastic nodules treated with CB13 than those treated with vehicle (Fig. 6B).

TNF- α mediates the increase in ceramide production induced by cannabinoid receptor activation. We tested the involvement of TNF- α in mediating the stimulation of ceramide synthesis induced by CB₁ and CB₂ agonists. The knockdown of TNF- α mRNA was obtained by transfecting cancer cells with a TNF- α -selective siRNA. The prevention of the CB13-stimulated increase in TNF- α levels in TNF- α -selective siRNA-transfected cells confirmed the knockdown of TNF- α mRNA after 48 hours of treatment (Fig. 6C). The lack of TNF- α function prevented the increase in ceramide production induced by ACEA or CB13 (Fig. 5A; Supplementary Fig. S3A and B). The administration of fumonisin B1 had no effect on the production of ceramide in the DLD-1 and HT29 cells transfected with TNF- α -selective siRNA and treated with CB₁/CB₂ receptor agonists (Fig. 5A). This clearly means that TNF- α is the main mediator of ceramide *de novo* synthesis following cannabinoid receptor activation in the DLD-1 and HT29 colon cancer cells. As a consequence,

the lack of TNF- α function in colon cancer cells prevented the ACEA/CB13-mediated activation of caspase-3 (Fig. 3A) and induction of apoptosis (Fig. 3B).

Discussion

There is growing evidence that cannabinoids may selectively target tumor cells by the activation of their membrane receptors, CB₁ and CB₂. However, the mechanisms underlying the antitumor effects of this activation are still not well understood, and experimental data suggest that these effects may be cell-type specific. The regulation of the RAS-mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and the phosphatidylinositol 3-kinase-AKT pathways and the stimulation of ceramide synthesis are among the mechanisms proposed to explain the antitumor effects of cannabinoids in different types of human cancer (reviewed in refs. 5, 6). Indeed, these pathways have been reported to be differently triggered depending on the tumor cell type investigated. In the present study, we report that both CB₁ and CB₂ cannabinoid receptor activation induces apoptosis in colon cancer cells, and this is mediated by the *de novo* synthesis of ceramide. Interestingly, we show for the first time that signaling through CB₁/CB₂ receptor increases ceramide production via a mechanism that involves TNF- α .

To our knowledge, only two previously published studies have investigated the expression of cannabinoid receptors in colorectal cancer and their involvement in mediating the antitumor effect of either the endocannabinoids anandamide/2-arachidonoylglycerol (34) or δ^9 -tetrahydrocannabinol (15). Ligresti et al. (34) found significant levels of CB₁ and CB₂ mRNA and protein expression in both normal and colorectal

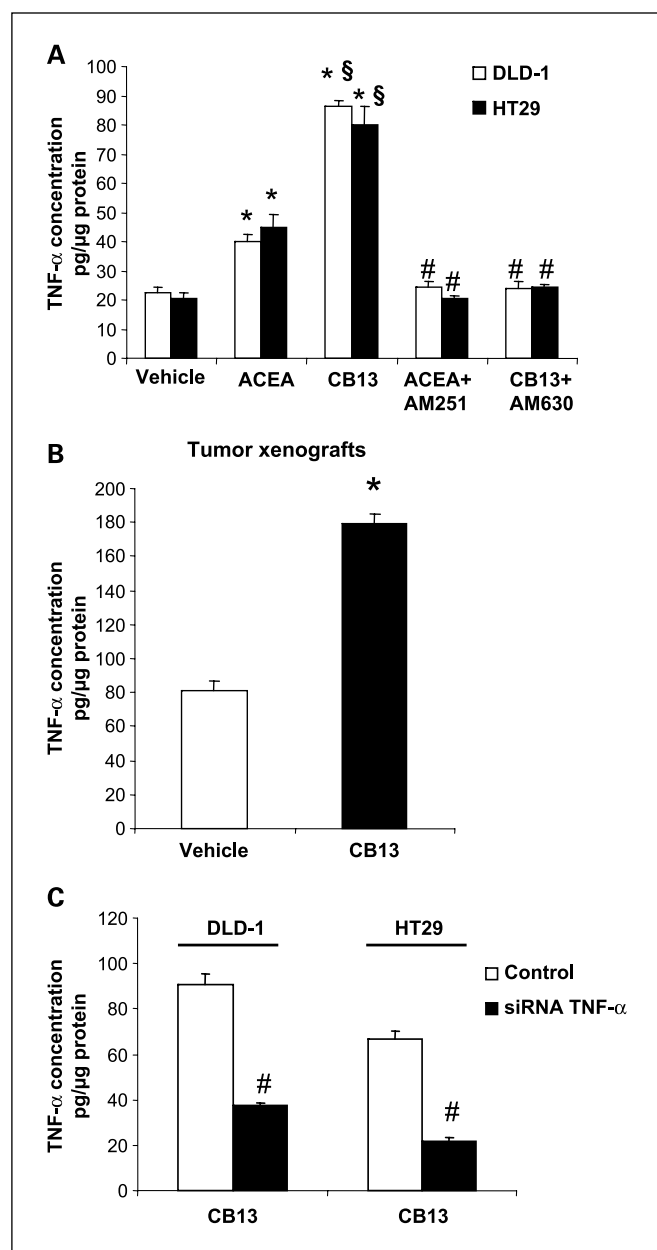


Fig. 6. TNF- α measurement. **A**, variations in TNF- α concentration in the DLD-1 and HT29 cells after 48 h of treatment with 100 nmol/L ACEA, 100 nmol/L CB13, ACEA + 100 nmol/L AM251 and CB13 + 100 nmol/L AM360. Columns, mean of five determinations; bars, SE. *, significant increase compared with vehicle treatment ($P < 0.05$); #, significant decrease compared with ACEA or CB13 treatment ($P < 0.05$); §, significantly different compared with treatment with ACEA ($P < 0.05$). **B**, variations in TNF- α concentration in tumors generated by s.c. injection of DLD-1 cells in BALB/c mice after treatment with CB13 (2.5 mg/kg/d). Columns, mean of five determinations; bars, SE. *, significant increase compared with vehicle treatment ($P < 0.05$). **C**, control of TNF- α knockdown after 48 h of treatment with CB13. Columns, mean of four determinations; bars, SE; #, significant decrease compared with control cells ($P < 0.05$).

cancer tissue and showed that the antiproliferative effect of anandamide and 2-arachidonoylglycerol in the Caco-2 and DLD-1 colon cancer cells is mediated by the activation of the CB₁ receptor. However, an antiproliferative role, even for CB₂ receptors in the DLD-1 cells, which express both cannabinoid receptors, has been proposed in the same study. Recently, Greenhough et al. (15) showed that δ^9 -tetrahydrocannabinol

can induce apoptosis in colorectal cancer cells by selective targeting of the CB₁ receptor through a mechanism involving the inhibition of the RAS-MAPK/ERK and phosphatidylinositol 3-kinase (PI3K)-AKT cell survival pathways and the increased expression of the proapoptotic protein BAD. These experiments were done on the SW480 colon cancer cell line, which expressed equal levels of CB₁ and CB₂ receptor expression. However, δ^9 -tetrahydrocannabinol has been shown to behave as a partial agonist at both CB₁ and CB₂ receptors, with less efficacy at the CB₂ ones (2). Therefore, these findings cannot be considered definitive and do not exclude a possible antitumor activity of highly selective CB₂ receptor ligands.

In the present study, we confirm that both human colorectal cancer specimens and the corresponding normal colonic mucosa express CB₁ and CB₂ receptors at the mRNA and protein levels as reported in previously published studies (15, 34). However, Western blot and immunohistochemical analyses showed a different expression pattern of the two receptors in either normal or neoplastic colon tissue. Indeed, the CB₁ receptor was mainly expressed by normal epithelial cells whereas CB₂ immunoreactivity was strongly positive in tumor epithelium. Because an anti-inflammatory role has been suggested for CB₂ receptor overexpression in colonic epithelium during the acute phase of inflammatory bowel diseases (33), it might be hypothesized that the induction of this receptor may represent a defensive mechanism of the colonic epithelium under pathologic conditions, such as chronic inflammation or malignant transformation. These data have to be taken into account when considering potential therapeutic applications of cannabinoids because only compounds with high selectivity for CB₂ receptor are likely to be effective as anticancer agents in humans.

To investigate the possible effects of CB₁ and CB₂ receptor activation on the inhibition of colon cancer growth, we used highly selective receptor ligands. This permits to maximally reduce any interference due to receptor-independent activities previously reported for both endogenous and natural cannabinoids (39). Moreover, we used these drugs at nanomolar concentrations, which are most likely comparable with those potentially detected in human serum after drug treatment, whereas natural cannabinoids such as δ^9 -tetrahydrocannabinol have been shown to induce cell death only at concentrations of 2.5 μ M and above (15, 40). We tested for the first time on tumor cells a newly synthesized CB₂ agonist, CB13, which has been shown to have higher CB₂ selectivity than the commercially available CB₂ agonists (18). We found that ACEA and, more efficiently, CB13 administration to DLD-1 and HT29 cells could induce a significant increase in caspase-3 activity and the number of tumor apoptotic cells. Moreover, we showed a significant cytotoxic effect of CB13 in four other colon cancer cells, HCT8, SW480, HCA7, and HCT15. To the best of our knowledge, this is the first report that signaling through the CB₂ receptor plays a major role in inducing apoptosis in colon cancer cells and exerts a remarkable growth-inhibiting effect in models of colon cancer *in vivo*. Previous observations have shown that the CB₂ receptor is involved in the antitumor effect of cannabinoids in other types of human tumors, such as gliomas (8), skin carcinomas (10), lymphomas (41), and pancreatic cancer (9). Altogether, these data may be of potential clinical importance because a new therapeutic approach to cancer might be on the basis of the use of nonpsychoactive

cannabinoid ligands (i.e., compounds without typical marijuana-like collateral effects due to CB₁ activation).

Ceramide is a well known proapoptotic lipid which has been shown to act as a second messenger of cannabinoid action (16, 17). CB₁ receptor activation induces ceramide accumulation in primary astrocytes and glioma cells through both sphingomyelin hydrolysis and ceramide *de novo* synthesis, thus playing an important role in regulating cell fate in neural disease and malignancy (16, 17). Recently, even CB₂ receptor activation has been shown to induce cell apoptosis through the stimulation of ceramide *de novo* synthesis in a number of human tumors, such as glioma (8, 16, 22), leukemia (23), and pancreatic cancer (9). In the present study we show that both CB₁ and CB₂ receptor activation stimulated ceramide synthesis in colon cancer cells, and its abrogation by the ceramide synthase inhibitor fumonisin B1 prevented the induction of apoptosis that occurred after the administration of ACEA and CB13. Moreover, ceramide expression was significantly higher in tumor xenografts treated with CB13 than those treated with vehicle. Therefore, these data point to ceramide as an important mediator of antitumor activity of cannabinoids even in colorectal cancer.

Some of the downstream targets of ceramide involved in cannabinoid-induced apoptosis have been recently identified. Carracedo et al. (9, 22) have shown that cannabinoid-induced ceramide synthesis in glioma and pancreatic cancer cells led to cell apoptosis through the up-regulation of the stress-regulation protein p8 and the endoplasmic reticulum stress-related genes *ATF-4* and *TRB3*. On the contrary, little is known about the signaling pathways underlying the promotion of ceramide synthesis through cannabinoid receptor activation. Experimental studies have provided evidence for the pivotal role of ceramide in transmitting some of the functional responses induced by TNF- α in adipocytes and neural cells (27, 28, 42).

Moreover, it has been shown that the cannabinoid system can modulate TNF- α production, with either suppressing (43, 44) or stimulating effects (45–48), depending on the type of cells investigated. In particular, the CB₁ receptor antagonist rimonabant can decrease the level of TNF- α in hepatic (45) and intestinal cells (46) whereas the agonist-specific stimulation of the CB₂ receptor has been shown to trigger the up-regulation of TNF- α mRNA in the promyelocytic cell line HL-60 (47) and in monocytes treated with *Echinacea* alkylamides (48). In the present study, we show for the first time that CB₁ and, more efficiently, CB₂ receptor activation induced TNF- α production in colon cancer cells and tumors generated in mice. Importantly, the knockdown of TNF- α function in the same cells abrogates the synthesis of ceramide and, consequently, the effects induced by CB₁ and CB₂ agonists on the induction of apoptosis. Therefore, TNF- α most likely plays a key role in the initialization of the antitumor activity induced by cannabinoid receptor activation through the induction of ceramide *de novo* synthesis.

In conclusion, the present study shows that the activation of the CB₁ and, more efficiently, of the CB₂ receptors exerts apoptotic effects in colorectal cancer. The lipid second messenger ceramide seems to play a major role in this process. Our data have unveiled, for the first time, that TNF- α acts as a link between cannabinoid receptor activation and ceramide production. The fact that selective targeting of CB₂ receptor results in colorectal tumor growth inhibition is of potential clinical interest for future cannabinoid-based anticancer therapies because the use of CB₂-selective ligands is not linked to the typical marijuana-like psychoactive effects of CB₁ activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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