Possible Endocannabinoid Control of Colorectal Cancer Growth

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Background & Aims: The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) inhibit cancer cell proliferation by acting at cannabinoid receptors (CBRs). We studied (1) the levels of endocannabinoids, cannabinoid CB1 and CB2 receptors, and fatty acid amide hydrolase (FAAH, which catalyzes endocannabinoid hydrolysis) in colorectal carcinomas (CRC), adenomatous polyps, and neighboring healthy mucosa; and (2) the effects of endocannabinoids, and of inhibitors of their inactivation, on human CRC cell proliferation.

Methods: Tissues were obtained from 21 patients by biopsy during colonoscopy. Endocannabinoids were measured by liquid chromatography-mass spectrometry (LC-MS). CB1, CB2, and FAAH expression were analyzed by RT-PCR and Western immunoblotting. CRC cell lines (CaCo-2 and DLD-1) were used to test antiproliferative effects.

Results: All tissues and cells analyzed contain anandamide, 2-AG, CBRs, and FAAH. The levels of the endocannabinoids are 3- and 2-fold higher in adenomas and CRCs than normal mucosa. Anandamide, 2-AG, and the CBR agonist HU-210 potently inhibit CaCo-2 cell proliferation. This effect is blocked by the CB1 antagonist SR141716A, but not by the CB2 antagonist SR144528, and is mimicked by CB2-selective, non CB2-selective, agonists. In DLD-1 cells, both CB2 and CB2 receptors mediate inhibition of proliferation. Inhibitors of endocannabinoid inactivation enhance CaCo-2 cell endocannabinoid levels and block cell proliferation, this effect being antagonized by SR141716A. CaCo-2 cell differentiation into noninvasive cells results in increased FAAH expression, lower endocannabinoid levels, and no responsiveness to cannabinoids.

Conclusions: Endocannabinoid levels are enhanced in transformed colon mucosa cells possibly to counteract proliferation via CBRs. Inhibitors of endocannabinoid inactivation may prove useful anticancer agents.

Numerous experimental data indicate that the activation of the endogenous cannabinoid system might represent a potential strategy for the development of new anticancer drugs.1,2 First, the psychoactive principle of Cannabis sativa and marijuana, Ñ2-tetrahydrocannabinol,3 is known to act mostly by stimulating 2 specific receptors subtypes, the cannabinoid CB1 and CB2 receptors4, and was reported in the past5 and more recently6 to have antineoplastic activity in vivo and in vitro.1,2 Second, endogenous agonists of the cannabinoid receptors (CBRs), i.e., N-arachidonoyl-ethanolamine (AEA; anandamide),7 2-arachidonoyl-glycerol (2-AG),8,9 and noladin ether,10 or their metabolically stable synthetic analogs, were found to inhibit, mostly via CB1 receptors, the proliferation of breast and prostate cancer cells in vitro11,12 and of rat thyroid cells transformed by the product of the K-ras oncogene in vivo.13 Finally, stimulation of the 2 CBR subtypes has been found to influence the expression of various genes involved in cell survival, proliferation, and apoptosis via interference with cAMP- and ceramide-mediated signalling, mitogen-activated protein kinases and phosphatidyl-inositol-3-kinase (see Guzman et al.14 for review).

Apart from the proposed role of CBRs in the control of cancer cell growth, transformation, and death,2,14 there are at least 3 reasons why the endocannabinoids might be involved in the control of colorectal cancer cell proliferation. First, both AEA and 2-AG are good substrates for cyclooxygenase 2 (COX-2), which seems to play a major role in the development of colorectal carcinoma (CRC).15,16 Because no molecular target has been reported to date for endocannabinoid COX-2 metabolites, it is possible that AEA, and particularly the more abundant 2-AG, might exert some of their biologic effects, including inhibition of CRC growth, via inhibition, by

Abbreviations used in this paper: AEA, arachidonoyl-ethanolamide (anandamide); 2-AG, 2-arachidonoyl-glycerol; CBR, cannabinoid receptors; CRC, colorectal carcinoma; COX-2, cyclooxygenase 2; FAAH, fatty acid amide hydrolase; LC-MS, liquid chromatography-mass spectrometry. © 2003 by the American Gastroenterological Association 0016-5085/03/$30.00 doi:10.1016/S0016-5085(03)00881-3
substrate competition, of COX-2–mediated formation of prostaglandins.\textsuperscript{16} Second, it has been reported recently\textsuperscript{17} that a particular class of lysophosphatic acids, molecules known to play a major role in the development of cancer and to be overproduced in several types of tumors, including CRC,\textsuperscript{18,19} might serve as biosynthetic precursors for 2-AG. Therefore, this possibly antiproliferative compound\textsuperscript{11,12} is likely to be overproduced in CRC as compared with normal colon tissue. Finally, the ras family of oncogenes plays a crucial role in the onset and growth of CRC,\textsuperscript{20} and we have recently reported that an endocannabinoid analogue inhibits the proliferation of v-K-ras–transformed thyroid cells by blocking the activity of p21ras, the protein encoded by ras.\textsuperscript{13}

Based on this background, we have decided to investigate whether endocannabinoids, their receptors, and one of the enzymes deputed to their inactivation, the fatty acid amide hydrolase (FAAH), are present, and with what possible biologic function, in CRCs as well as in colorectal adenomatous polyps that are known to progress into CRC. We compared the levels of AEA, 2-AG, CB\textsubscript{1}, CB\textsubscript{2}, and FAAH in normal colon mucosa to those in transformed mucosa (adenomas and carcinomas). Furthermore, we studied the effect of the endocannabinoids, of selective CB\textsubscript{1} and CB\textsubscript{2} receptor stimulation, and of selective inhibitors of endocannabinoid inactivation (to augment AEA and 2-AG endogenous levels) on the proliferation of two human CRC cell lines: (1) the CaCo-2 cells, which are widely used for studies on this type of cancer and undergo differentiation when in culture; and (2) DLD-1 cells, which, unlike CaCo-2 cells, do not differentiate in culture. We report data pointing to a tonic limiting action by endocannabinoids and CBRs on the growth of CRC.

Materials and Methods

Drugs

AEA and 2-AG were purchased from Cayman Chemicals, and ACEA, Met-Fluoro-anandamide, and BML-190 from Tocris. HU-210 was a kind gift from Dr. Ron Mechoulam, Hebrew University of Jerusalem, and SR144528 were donated by Sano Recherche. Indomethacin N-methyl-ester was obtained from Sigma. VDM-11, VDM-13, and arachidonoyl-serotonin were synthesized from the corresponding amines and arachidonoyl-chloride, as described previously.\textsuperscript{21}

Biopsy

Biopsy specimens were obtained in agreement with current Italian health care rules, by means of biopsy forceps during colonoscopy on both healthy and cancer tissue in 9 patients affected with left-sided colon carcinoma (average age 64.5 ± 10 years, 3 males, 6 females; mitosis = 1.35 ± 0.33; grading = 2.44 ± 0.52; nuclear pleiomorphism 2.55 ± 0.52; means ± SD) and on healthy tissue and adenomatous polyps in 12 patients affected with colonic adenomas (average age 59.9 ± 14 years, 10 males, 2 females). A small piece (15–20 mg wet weight) from the head of each polyp removed by snare polypectomy and aliquots of each biopsy sample were kept at −80°C until processing. Adenomatous polyps and aliquots of all samples were stored in formalin for histology to evaluate tumor grading, mitoses for high-power field, and nuclear pleiomorphism.

Cell Culture and Proliferation and Differentiation Assays

Caco-2 cells were grown in Dulbecco’s modified Eagle medium supplemented with 2 mmol/L L-glutamine, 1% non-essential amino acids, and 10% fetal calf serum (FCS). DLD-1 cells were grown in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine and 10% FCS. Sucrase activity was assessed by the method of Dahlquist\textsuperscript{22} by measuring the glucose released from saccharose by the enzyme under standardized condition. The sucrase activity was expressed as 1 unit = 1 μmol of glucose released/min at 37°C at pH 7. Cell proliferation assays were carried out in 6-well dishes containing subconfluent cells (5 × 10\textsuperscript{4} cells). Three hours after cell seeding, test substances were added in medium and then daily at each change of medium. After 4 days, cells were treated with trypsin and counted by a hemocytometer. Cell viability was assessed by trypan blue, and no significant decrease was observed with up to 10 μmol/L anandamide. DNA fragmentation of CaCo-2 cells treated for 72 hours with met-fluoro-anandamide (Tocris) was analyzed by flow cytometry using FACSscan (Becton Dickinson).\textsuperscript{11}

Endocannabinoid Measurement

Tissues or cells were dounce-homogenized with chloroform/methanol/Tris-HCl 50 mmol/L, pH 7.4 (1/1/1 by volume), containing 5 pmol of δ\textsubscript{2}-anandamide and 50 pmol of δ\textsubscript{2}-2-AG (Cayman Chemicals) as internal standards. Lipid-containing organic phase was dried down, weighed, and purified by open-bed chromatography on silica gel and analyzed by liquid chromatography (LC)-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) (LC-APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu LCMS-2010 quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected ion-monitoring (SIM) mode as described previously.\textsuperscript{23} Anandamide and 2-AG quasimolecular ions were quantified by isotope dilution with the above-mentioned deuterated standards and their amounts in pmols normalized per milligram of lipid extract.\textsuperscript{23} Data were statistically evaluated by ANOVA followed by the Bonferroni’s test (as per StatMost).
Reverse-Transcriptase Polymerase Chain Reaction

The expression of messenger RNA (mRNA) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COX-2, FAAH, CB1, and CB2 receptors was examined by reverse transcription coupled to the polymerase chain reaction (RT-PCR). The procedures have been widely described in our previous studies.11–13 The PCR cycles were 35 for CB1, CB2, FAAH, and COX-2 and 28 for GAPDH, which were observed to be optimal and in the linear portion of the amplification curve. The specific human oligonucleotides were synthesized on the basis of cloned human cDNA sequences of GAPDH, COX-2, FAAH, CB1, and CB2. For GAPDH, the primer sequences were 5′-CCCTCCTAGGCTTCAAATCATTGGT-3′ (nt 208-233; sense) and 5′-GAGGAGGCATCCAAGTCTTCTGT-3′ (nt 655-677; antisense). The COX-2 sense and antisense primers were 5′-TGGGAAGGCTTCTCTAACATCCCTCTCT-3′ (nt 125-132) and 5′-CTTTGACTGTGGGAAGCTTCTCT-3′ (nt 246-254), respectively. The FAAH sense and antisense primers were 5′-GTGTTGCTG(T/G)AACCCCCATGCTG-3′ (nt 469-475) and 5′-TCCACCTCCGCGATGAAACCCAGACA-3′ (nt 561-569), respectively. The CB1 sense and antisense primers for CB2 were 5′-GATGTCTTTGGGAAAGTGAACAGC-3′ (nt 365-373) and 5′-AGACGTGTCTGTGGACACAGACATGG-3′ (nt 460-468), respectively. For CB2, the primer sequences were 5′-TTTCTCCACTGTCCCTCC-3′ (nt 182-188; sense) and 5′-AGTTGATGAGCCACAGCATG-3′ (nt 285-291; antisense). The expected sizes of the amplicons were 470 bp for GAPDH, 388 bp for COX-2, 300 bp for FAAH, 309 bp for CB1, and 329 bp for CB2. In the presence of contaminant genomic DNA, the expected size of the amplicons would be 1062 bp for GAPDH, 1668 bp for COX-2, and 1335 bp for FAAH, 329 bp for CB2. In the presence of contaminant genomic DNA, the expected size of the amplicons would be 1062 bp for GAPDH, 1668 bp for COX-2, and 1335 bp for FAAH, respectively. The GAPDH housekeeping gene expression was used to evaluate any variation in the RNA content and cDNA synthesis in the different preparations. No PCR products were detected when the reverse transcriptase step was omitted (data not shown).

Western Immunoblotting

Western immunoblotting analysis was used to determine the presence of the CB1 and CB2 proteins and carried out as described in detail previously.11–13 Antibodies (both from Cayman Chemicals, Ann Arbor, MI) were used at a dilution of 1:333 for CB1 and 1:250 for CB2. Control of specificity was performed by preabsorbing the antibody with the homologous antigen at a concentration of 8 μg/mL of antibody solution.

Anandamide Uptake Assays

Confluent Caco-2 cells (plated in 6-well dishes, 150,000 cells per dish) take up [14C]AEA (5.0 μmol/L, 20,000 cpm) from serum-free medium in a time- and temperature-dependent manner (t1/2 = 3.5 minutes, uptake at 37°C = uptake at 4°C = 48.3 ± 4.1% of total uptake). The effect of compounds on [14C]AEA uptake was studied as described previously.21 Cells were incubated with [14C]AEA for 5 minutes at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [14C]AEA in the incubation media after extraction with CHCl3/CH3OH 2:1 (by volume), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells.

Anandamide Hydrolysis Assays

The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously,21 using membranes prepared from cells incubated with the test compounds and [14C]AEA (10 μmol/L, 40,000 cpm) in 50 mmol/L Tris-HCl, pH 9, for 30 minutes at 37°C. [14C]Ethanolamine produced from [14C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl3/CH3OH 2:1 (by volume).

Binding Assays

Displacement assays for CB1 receptors were carried out by using [3H]SR141716A (0.4 nmol/L, 55 Ci/mmol; Amersham) as the high-affinity ligand on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Wilmington, MA) and in the presence of 100 μmol/L PMSF.21 Specific binding was calculated with 1 μmol/L SR141716A and was 84%. Data are expressed as the Kᵢ, calculated using the Cheng-Prusoff equation from the concentration exerting 50% inhibition of AEA uptake (IC₅₀).

Results

Endocannabinoid Levels and CB1, CB2, and FAAH Expression in Human Colorectal Tissues

We found that human colon mucosa tissues contain both AEA and 2-AG (Figure 1A), as determined by using an ultrasensitive LC-MS technique, and express mRNA transcripts of the size expected for CB1 and CB2 receptors as well as FAAH (Figure 1B), as determined by RT-PCR. The finding of CB1 receptors was also confirmed by Western immunoblotting of proteins from biopsy specimens of normal colon mucosa (not shown). The levels of both AEA and 2-AG increased when passing from normal mucosa to transformed mucosa (Figure 1A). Although the levels of endocannabinoids in colorectal adenomas and carcinomas could not be determined in the same set of patients, it was possible to observe a stronger increase of the amounts of 2-AG and, particularly, AEA (3-fold vs. 2-fold, respectively) in adenomatous polyps than in CRC tissue as compared with healthy mucosa. Tissues from both normal and transformed mucosa yielded similar amounts of extracted lipids per gram of wet weight, with normal mucosa, adenomas, and CRC
tissue containing 130 ± 7, 124 ± 5, and 119 ± 4 mg lipids/g wet tissue weight (means ± SEM, n = 21, 12, and 9, respectively), respectively. Therefore, the ranking of endocannabinoid content in the 3 types of tissues (adenomas>CRC>normal mucosa) did not change when the amounts were expressed as pmol/g wet tissue weight (Figure 1A, inset). No consistent differences between the levels of the expression of CB1, CB2, and FAAH (the former as assessed by both RT-PCR and immunoblot) were found between normal and CRC tissue (Figure 1B and data not shown).

**CRC Cell Differentiation and/or Proliferation In Vitro**

CaCo-2 cells reached confluence after about 7 days from cell seeding and, starting from the 12th day in culture, started differentiating as assessed by the progressive synthesis of sucrase (Figure 2A). Both AEA and 2-AG significantly inhibited the growth of undifferentiated CaCo-2 cells with IC50 in the submicromolar range (Figure 2B). No toxicity to cells, as assessed by the trypan blue method, and no effect on apoptosis, as assessed by FACScan, was observed up to 5 μmol/L AEA (not shown). The CBR agonist HU-210 inhibited CaCo-2 cell proliferation more potently than the 2 endocannabinoids. Two agonists selective for CB1 vs. CB2 receptors, i.e., arachidonoyl-chloro-anandamide (ACEA) and N-arachidonoyl-dopamine (NADA), also inhibited CaCo-2 cell proliferation, whereas the CB2-selective agonist BML-190 was inactive (Figure 2C). Importantly, the effect of AEA, HU-210, and NADA was antagonized by the selective CB1 receptor antagonist SR141716A (0.2 μmol/L) but not by the selective CB2 receptor antagonist SR144528 (0.2 μmol/L, Figure 2D and data not shown). During the course of differentiation, the responsiveness of CaCo-2 cells to AEA and HU-210 changed dramatically, the proliferation of differentiated cells being almost insensitive to treatment with these 2 compounds (Table 1).

We also assessed the effect of AEA, 2-AG, HU-210, and BML-190 on the nondifferentiating DLD-1 cells. In this case, stimulation of both CB1 and CB2 receptors led to inhibition of cell growth, although with lower efficacy as compared with CaCo-2 cells (Table 1). The effect of HU-210 was counteracted by both SR141716A and SR144528 (0.2 μmol/L, Table 1).

The selective COX-2 inhibitor indomethacin N-methyl ester (0.1-25 μmol/L) inhibited undifferentiated CaCo-2 cell proliferation (Table 1). This effect of was not additive with that exerted by 2-AG, which instead occluded the antiproliferative effect indomethacin N-methyl ester (Table 1). The COX-2 inhibitor also blocked the proliferation of DLD-1 cells, and this effect was antagonized by SR141716A (Table 1).

**Presence of CBRs in CRC Cells in Culture**

By using RT-PCR, we found that CaCo-2 cells express the CB1 receptor, whereas no evidence for the presence of CB2 mRNA transcripts was found (Figure 3A and data not shown). Western immunoblotting con-
confirmed that CB1 is expressed in these cells, in that 3 immunoreactive bands, sensitive to saturation with the blocking peptide, were found with molecular weight very similar to those found in rat brain homogenates (Figure 3C) and corresponding to the truncated, native, and glycosylated forms of the CB1 receptor. Overall, the levels of CB1 receptors in CaCo-2 cells appeared to remain constant upon differentiation of the cells, although the amounts of the native form (—53 kilodaltons) of the receptor were significantly lower than in undifferentiated cells (Figure 3C). DLD-1 cells were found to express both CB1 and CB2 mRNA and protein, although CB1 receptors appeared to be less abundant in these cells than in CaCo-2 cells (Figure 3A and C).

Endocannabinoids and Their Inactivation in CRC Cells

By using again our sensitive LC-MS analytical method, we found that undifferentiated CaCo-2 cells contain measurable levels of both AEA (Table 2). We also found that these cells exhibit FAAH activity (93.6 ± 11.4 pmol mg protein⁻¹ minute⁻¹, mean ± SD, n = 3) and, like DLD-1 cells, express FAAH mRNA (Figure 3B). Intact, undifferentiated CaCo-2 cells also clear [¹⁴C]AEA from the incubation medium in a temperature-dependent manner (67.0 ± 3.2 pmol minutes⁻¹ per 10⁶ cells, corresponding to 111.7 ± 5.3 pmol minutes⁻¹ mg protein⁻¹, mean ± SD, n = 3). The level of expression of FAAH mRNA in CaCo-2 cells appeared to increase upon differentiation of the cells (Figure 3B), thus explaining why the amounts of endocannabinoids in differentiated cells were significantly lower than in undifferentiated cells (Table 2).

The effect of the 2 selective AEA uptake inhibitors VDM11 and VDM13 and of the selective FAAH inhibitor arachidonoyl-serotonin were examined on the uptake and hydrolysis, respectively, of [¹⁴C]AEA by CaCo-2 cells. VDM11 and VDM13 efficiently inhibited [¹⁴C]AEA uptake with similar IC₅₀ values around 3 μmol/L (Figure 4A and data not shown). Arachidonoyl-serotonin instead inhibited [¹⁴C]AEA hydrolysis with an IC₅₀ ~9 μmol/L (Figure 4B).

Figure 2. CaCo-2 cell proliferation and differentiation and the effect of cannabimimetics. (A) Growth of CaCo-2 cells in culture and sucrase production as an index of differentiation. (B) Effect of anandamide, 2-arachidonoyl-glycerol (2-AG), and the potent CB₁/CB₂ agonist HU-210 on the proliferation of undifferentiated (days 0–5) CaCo-2 cells. (C) Effect of the selective CB₁ agonists arachidonoylchloroethanolamide (ACEA) and N-arachidonoyl-dopamine (NADA) and of the selective CB₂ agonist BML-190 on the proliferation of undifferentiated (days 0–5) CaCo-2 cells. (D) Effect of the selective CB₁ antagonist SR141716A on the antiproliferative action of HU-210, anandamide, and NADA. *P < 0.01 vs. control, by ANOVA. In B–D, data are expressed as percentage of control cell proliferation (100 × final treated cell number — initial cell number/final control cell number) and are means of n = 3 experiments carried out in duplicate. In B and C, SEM bars are not shown for the sake of clarity and were never higher than 5% of the means.
**Table 1.** Effect of Various Cannabinimetic Agents and of Indomethacin N-Methyl Ester on the Proliferation of CRC Cells in Culture

<table>
<thead>
<tr>
<th></th>
<th>DLD-1</th>
<th>CaCo-2 (days 1–5)</th>
<th>CaCo-2 (days 14–16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEA (2.5 μmol/L)</td>
<td>59.8 ± 5.4</td>
<td>27.0 ± 3.1</td>
<td>96.0 ± 2.2</td>
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<tr>
<td>2-AG (1 μmol/L)</td>
<td>75.0 ± 3.1</td>
<td>41.6 ± 1.5</td>
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<tr>
<td>2-AG (2.5 μmol/L)</td>
<td>59.3 ± 3.8</td>
<td>23.8 ± 0.1</td>
<td>NT</td>
</tr>
<tr>
<td>HU-210 (0.1 μmol/L)</td>
<td>100.2 ± 1.5</td>
<td>18.3 ± 1.9</td>
<td>90.6 ± 2.8</td>
</tr>
<tr>
<td>HU-210 (1 μmol/L)</td>
<td>57.1 ± 3.5</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>HU-210 (1 μmol/L) + SR141716A (0.2 μmol/L)</td>
<td>79.8 ± 2.2</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>HU-210 (1 μmol/L) + SR144528 (0.2 μmol/L)</td>
<td>88.7 ± 4.2</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>BML-190 (1 μmol/L)</td>
<td>66.5 ± 3.1</td>
<td>90.7 ± 4.5</td>
<td>NT</td>
</tr>
<tr>
<td>BML-190 (1 μmol/L) + SR144528 (0.2 μmol/L)</td>
<td>90.5 ± 4.1</td>
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<td>NT</td>
</tr>
<tr>
<td>INDO (0.1 μmol/L)</td>
<td>100.0 ± 5.2</td>
<td>74.0 ± 0.1</td>
<td>NT</td>
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<tr>
<td>INDO (1 μmol/L)</td>
<td>99.0 ± 3.5</td>
<td>74.2 ± 1.5</td>
<td>NT</td>
</tr>
<tr>
<td>INDO (10 μmol/L)</td>
<td>63.0 ± 4.2</td>
<td>62.6 ± 3.3</td>
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<tr>
<td>INDO (25 μmol/L)</td>
<td>22.0 ± 0.9</td>
<td>10.3 ± 2.8</td>
<td>NT</td>
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<tr>
<td>INDO (25 μmol/L) + 2-AG (1 μmol/L)</td>
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<td>29.6 ± 3.8</td>
<td>NT</td>
</tr>
<tr>
<td>INDO (25 μmol/L) + 2-AG (2.5 μmol/L)</td>
<td>NT</td>
<td>17.7 ± 2.1</td>
<td>NT</td>
</tr>
<tr>
<td>INDO (25 μmol/L) + SR141716A (0.2 μmol/L)</td>
<td>61.5 ± 3.1</td>
<td>NT</td>
<td>NT</td>
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</table>

**Note.** Data are expressed as percentage of control cell proliferation (100 ×; final treated cell number – initial cell number/final control cell number) and are means of n = 3 experiments carried out in duplicates. Both undifferentiated (days 1–5) and differentiated (days 14–16) CaCo-2 cells were used.

*a*ANOVA, ANOVA followed by the Bonferroni test.

**Effect of Selective Inhibitors of Endocannabinoid Inactivation on CaCo-2 Cell Proliferation**

VDM11, VDM13, and arachidonoyl-serotonin were also evaluated for their effect on undifferentiated CaCo-2 cell proliferation. We found that the 3 compounds inhibited proliferation with IC_{50} values almost identical to those observed for the inhibition of [1^4C]AEA uptake and hydrolysis, respectively (Figure 4A and B and data not shown). Importantly, the effect on cell proliferation of a 10 μmol/L concentration of the 3 inhibitors was antagonized by SR141716A (0.2 μmol/L; Figure 4A and B). Accordingly, we found that 24-hour incubation of undifferentiated CaCo-2 cells with either VDM-11 (10 μmol/L) or arachidonoyl-serotonin (10 μmol/L) led to a significant increase of endocannabinoid levels (Table 2).

**COX-2 Expression in CRC Cell Lines**

A messenger RNA transcript for COX-2 was detected both in undifferentiated CaCo-2 cells and, to a much smaller extent, DLD-1 cells (Figure 5). The level of expression of COX-2 decreased significantly when CaCo-2 cells underwent differentiation (Figure 5).

**Discussion**

We found that human colon mucosa tissues contain both AEA and 2-AG and express CB_1 and CB_2 receptors as well as FAAH. The endocannabinoids and FAAH previously have been described to occur in mouse and rat whole colon, but we found here that the levels of both AEA and 2-AG increase dramatically when passing from normal mucosa to adenomatous polyps and then slightly decrease in CRC tissue. These changes are likely to result in corresponding changes in endocannabinoid tissue concentrations. In fact, considering that, on average, 1 g (and, hence, ~1 mL) of wet tissue weight yields ~125 mg of extracted lipids, it can be calculated that AEA concentrations augment from around 75 pmol/g (i.e., ~75 nmol/L) to 143 and 236 pmol/g (i.e., ~143 nmol/L and ~236 nmol/L) when passing from healthy mucosa to CRC and adenomatous polyps, respectively. Therefore, AEA concentrations are increased to concentrations well above the threshold of CB_1 activation by this lipid (the reported Ki values for CB_1 receptors are in the 40–200 nmol/L range). Regarding 2-AG, the concentration of this compound augment from about 32 μmol/L, in normal tissue, to 71 and 93 μmol/L, respectively, in CRC and adenomas. Although the levels of 2-AG in healthy mucosa appear to be already sufficient to tonically activate CB_1 receptors (the reported Ki values for 2-AG for CB_1 receptors are in the 250–1200 nmol/L range), it must be kept in account that only a part of 2-AG found in tissues might be used as an endocannabinoid. In fact, this compound, unlike AEA, is also an intracellular intermediate of (phospho)glyceride metabolism and is released outside cells only in part to activate CBRs.
Several biochemical mechanisms might explain the enhancement of endocannabinoid levels in transformed human colon mucosa. First, an up-regulation of 1 of the enzymes responsible for arachidonate mobilization and phospholipid remodelling, the secretory phospholipase A2, has been described to occur in colorectal adenomas from familial adenomatous polyposis patients. 26 Second, elevated levels of lysophosphatidic acids, possibly also including the 2-arachidonate-containing species that serve as biosynthetic precursors for 2-AG,17 have been reported in patients with CRC. 19 These 2 events, albeit suggested to play a causative role in the onset and growth of tumors, via enhanced formation of the precursor for COX-2-catalyzed production of prostaglandins15 or stimulation of LPA receptors, 18 respectively, might lead at the same time to elevated levels of endocannabinoids with tumor-inhibitory activity.

To test the above hypothesis, we undertook a series of experiments aimed at investigating whether (1) endocannabinoids do inhibit colon cancer cell growth in vitro; (2) substances that inhibit endocannabinoid inactivation, and hence enhance the amounts of endocannabinoids produced by CRC cells in culture, also inhibit the proliferation of these cells; and (3) changes in endocannabinoid signaling occur in CRC cells also during their differentiation in culture. We used a widely employed cell line for the study of CRC, the CaCo-2 cells, which have the special feature of being capable of differentiating in culture after having reached confluence, thus becoming more similar to enterocytes.27 We found that

Table 2. Amounts of Endocannabinoids in Undifferentiated and Differentiated CaCo-2 Cells in Culture

<table>
<thead>
<tr>
<th></th>
<th>Anandamide</th>
<th>2-arachidonoyl-glycerol</th>
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<tr>
<td>Differentiated cells + vehicle</td>
<td>10.8 ± 3.0a</td>
<td>107.1 ± 3.5a</td>
</tr>
<tr>
<td>Undifferentiated cells + vehicle + VDM11</td>
<td>41.0 ± 7.6</td>
<td>208.0 ± 45.1</td>
</tr>
<tr>
<td>+ arachidonoyl-serotonin</td>
<td>70.0 ± 2.6a</td>
<td>400.0 ± 120.1a</td>
</tr>
</tbody>
</table>

NOTE. Table shows the effect of 24-hour cell treatment with vehicle (methanol, 0.1%) and VDM11 (10 μmol/L), and arachidonoyl-serotonin (10 μmol/L) of undifferentiated cells. Data are expressed as pmol/g wet cell weight and are means ± SD of n = 3 determinations. aP < 0.05 vs. undifferentiated cells + vehicle, as assessed by the Student t test.
AEA, 2-AG, and the ultra-potent CBR agonist HU-210 inhibited CaCo-2 cell proliferation with IC_{50} values in the submicromolar range and with relative potencies (HU-210 \gg AEA \cong 2-AG) that reflect their relative potencies at cannabinoid CB_{1} receptors. Three further observations strongly supported the involvement of this receptor subtype in endocannabinoid antiproliferative effects. First, 2 agonists selective for CB_{1} vs. CB_{2} receptors, i.e., arachidonoyl-chloro-anandamide (ACEA) and N-arachidonoyl-dopamine (NADA), the latter of which is much more stable to enzymatic hydrolysis than AEA, but less potent on CB_{1} receptors than AEA,\textsuperscript{28} inhibited CaCo-2 cell proliferation with the rank of potency expected from their relative affinity for CB_{1} receptors (ACEA \gg NADA), whereas the CB_{2}-selective agonist BML-190 was inactive. Second, the effect of AEA, HU-210, and NADA (which is also an agonist for the VR1 receptor for capsaicin\textsuperscript{28}) was entirely antagonized by the selective CB_{1} receptor antagonist SR141716A but not by the selective CB_{2} receptor antagonist SR144528. Finally, we found that CaCo-2 cells express the CB_{1} receptor, whereas no evidence for the presence of CB_{2} was found.

We also assessed the effect of the endocannabinoids and of HU-210 and BML-190 in another CRC cell line, the nondifferentiating DLD-1 cells. In this case, stimulation of both CB_{1} and CB_{2} receptors led to inhibition of cell growth, in agreement with the presence of both receptor subtypes in these cells. However, CB_{1} receptors appeared to be less expressed in DLD-1 cells than in CaCo-2 cells, and this finding, together with the observation that DLD-1 cells were clearly less responsive to cannabimimetics than CaCo-2 cells, might suggest that CB_{1} receptors are more important than CB_{2} receptors in causing blockade of CRC cell proliferation. In summary, we found that, depending on the CRC cell line, either selective CB_{1} receptor stimulation (as previously found for breast and prostate cancer cells\textsuperscript{11,12}) or activation of both CB_{1} and CB_{2} receptors causes inhibition of proliferation. These findings are in agreement with the presence of both CBR subtypes in colon normal mucosa and CRC (Figure 1B) and suggest that endocannabinoids, present in high amounts in CRCs and, particularly, colorectal adenomas, might function as endogenous inhibitors of cancer growth.

To further challenge this hypothesis, we started a series of experiments aimed at manipulating pharmacologically the endogenous levels of endocannabinoids in CRC cells without directly activating the CBRs and at evaluating whether these treatments lead to inhibition of...
Another approach that we used to gain further support to this hypothesis was to see whether, in CRC cells, responsiveness to (endo)cannabinoids and the extent of endocannabinoid signalling change during cell differentiation. To this purpose, we exploited the capability of CaCo-2 cells to differentiate in culture into enterocytes with a much lower degree of malignancy and invasiveness. First, we observed that AEA was only efficacious against undifferentiated cells. This finding might have been caused by the fact that cell differentiation was accompanied by an increase of FAAH expression, which is likely to lead to an enhanced AEA degradation in differentiated cells. However, because HU-210, which is not a substrate for FAAH, was inactive on differentiated cells, the increase of FAAH expression might not be the sole cause of the lack of activity of AEA on differentiated cells. We also analyzed the expression of CB1 receptors in differentiated CaCo-2 cells and found overall CB1 levels similar to those observed in undifferentiated cells. However, the amounts of the native form (~53 kilodaltons) of the receptor appeared to be significantly lower than in undifferentiated cells. It is, therefore, possible that the smaller antiproliferative effect of HU-210 in differentiated cells is due to a decrease of the levels of functionally active CB1 or, alternatively, to changes in CB1-coupled intracellular signalling events during CaCo-2 cell differentiation. We also measured the endocannabinoids in differentiated and undifferentiated CaCo-2 cells and found significantly lower amounts of both AEA and 2-AG, in agreement with the higher FAAH expression in differentiated cells and with our data obtained with arachidonoyl-serotonin (Table 2).

These findings, as well as preliminary data obtained in our laboratory, indicating no significant change in the expression of monoacylglycerol lipase (another 2-AG metabolizing enzyme) during CaCo-2 cell proliferation, suggest that FAAH plays an important role in limiting the levels of both AEA and 2-AG in CaCo-2 cells. It is worthwhile noting that, in agreement with our findings, in human breast cancer cells, whose proliferation is blocked by endocannabinoids via CB1 receptors, FAAH has been shown very recently to be expressed in up to 30-fold higher levels in those cell lines with a lower degree of invasiveness and malignancy. At any rate, the overall reduction of both the levels and the antiproliferative effects of endocannabinoids in differentiated (and less malignant) vs. undifferentiated (and more malignant) CaCo-2 cells suggests that, also in vitro, endocannabinoid signalling is regulated depending on colorectal cell differentiation and supports our hypothesis that AEA and 2-AG might act as endogenous CRC growth inhibitors.
The higher sensitivity to AEA and 2-AG of undifferentiated CaCo-2 cells as compared with DLD-1 and differentiated CaCo-2 cells might be also explained by the additional action of the 2 endocannabinoids, only in the former cells, on a target different from CB1. This target might be COX-2 because (1) AEA and 2-AG compete efficaciously with arachidonic acid as COX-2 substrates\(^{16}\) and might occlude the formation of protumoral prostaglandins, and (2) COX-2 is virtually absent in differentiated CaCo-2 and DLD-1 cells (Figure 5).\(^{27,34}\)

We found that the selective COX-2 inhibitor indo-mentacin N-methyl ester\(^{35}\) inhibited undifferentiated CaCo-2 cell proliferation at concentrations (\(IC_{50} = 14 \text{ } \mu \text{mol/L}\)) much higher than those required to inhibit the enzyme in vitro (\(IC_{50} = 0.04 \text{ } \mu \text{mol/L}\)). This antiproliferative effect was occluded by 2-AG, thus suggesting that the 2 substances share, at least in part, a similar mechanism of action. However, the COX-2 inhibitor (1) was also active on DLD-1 cells (which express little COX-2) where its effect was antagonized by SR141716A and (2) acted as a CB1 receptor ligand in binding assays carried out with rat brain membranes (\(K_i = 2.8 \pm 0.3 \text{ } \mu \text{mol/L, mean} \pm \text{SD, n = 3}\)). This indicates that, like 2-AG, indomethacin N-methyl ester inhibits CRC cell proliferation by stimulating CBRs. Hence, the reason why (endocannabinoids are less potent in DLD-1 cells is not because they cannot act by inhibiting COX-2 expression, as suggested by the observation that HU-210, which does not inhibit COX-2, is also much less efficacious in the same cells (Table 1).

In conclusion, we have shown that endocannabinoids (1) are overproduced in cancerous and, particularly, pre-cancerous (i.e., adenomas) colon tissue and (2) exert a growth-inhibitory effect on CRC cells in culture, in which the extent of their action and levels seems to depend on the degree of differentiation (and malignancy/invasiveness) of these cells. The antiproliferative effects of the endocannabinoids are exerted in a large part by stimulation of CBRs, which are expressed in both colorectal mucosa and CRC cells. However, these compounds might act also by inhibiting COX-2, a possibility that, although not supported by our present data, deserves further investigation. Whatever their mechanism of action, endocannabinoids can be regarded as potential endogenous tumor growth inhibitors as well as possible markers for cancer cells. This hypothesis is strengthened by a recent preliminary study\(^{36}\) showing that AEA levels are increased in other tumors, including those whose growth was previously shown to be inhibited by endocannabinoids.\(^{11,12}\)

Metabolically stable substances that act by stimulating CBRs directly might exert anticancer actions in this as well as other type of tumors.\(^{1,2}\) However, in view of (1) the possible multiple mechanisms of action of endocannabinoids, (2) the potential undesired psychotropic effect of CB1 receptor agonists, and (3) the tonic inhibition on cancer growth suggested here for endocannabinoids, substances that act selectively by enhancing further the tumor levels of AEA and 2-AG, such as inhibitors of their cellular uptake and enzymatic hydrolysis, might provide for a more efficacious and tolerable therapeutic strategy against not only CRC but also other types of cancer.\(^{2,30}\) In support of this possibility, we have found in a separate study (V. Di Marzo, G. Portella and M. Bifulco, manuscript in preparation) that the growth of transformed thyroid cells in athymic mice\(^{13}\) is inhibited efficaciously by the AMT inhibitor VDM11 and by the FAAH inhibitor arachidonyl-serotonin via enhancement of tumoral endocannabinoid levels.

References

12. Melck D, De Petrocellis L, Orlando P, Bisogno T, Lazzza C, Bifulco M, Di Marzo V. Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition


