Opposing Actions of Endocannabinoids on Cholangiocarcinoma Growth

RECRUITMENT OF Fas AND Fas LIGAND TO LIPID RAFTS*

Cholangiocarcinomas are devastating cancers of biliary origin with limited treatment options. Modulation of the endocannabinoid system is being targeted to develop possible therapeutic strategies for a number of cancers; therefore, we evaluated the effects of the two major endocannabinoids, anandamide and 2-arachidonylglycerol, on numerous cholangiocarcinoma cell lines. Although anandamide was antiproliferative and proapoptotic, 2-arachidonylglycerol stimulated cholangiocarcinoma cell growth. Specific inhibitors for each of the cannabinoid receptors did not prevent either of these effects nor did pretreatment with pertussis toxin, a Gi/o protein inhibitor, suggesting receptors did not prevent either of these effects nor did pretreatment with pertussis toxin, a Gi/o protein inhibitor, suggesting that anandamide and 2-arachidonylglycerol did not exert their diacritic effects through any known cannabinoid receptor or through any other Gi/o protein-coupled receptor. Using the lipid raft disruptors methyl-β-cyclodextrin and filipin, we demonstrated that anandamide, but not 2-arachidonylglycerol, requires lipid raft-mediated events to inhibit cellular proliferation. Closer inspection of the lipid raft structures within the cell membrane revealed that although anandamide treatment had no observable effect 2-arachidonylglycerol treatment effectively dissipated the lipid raft structures and caused the lipid raft-associated proteins lyn and flotillin-1 to disperse into the surrounding membrane. In addition, anandamide, but not 2-arachidonylglycerol, induced an accumulation of ceramide, which was required for anandamide-induced suppression of cell growth. Finally we demonstrated that anandamide and ceramide treatment of cholangiocarcinoma cells recruited Fas and Fas ligand into the lipid rafts, subsequently activating death receptor pathways. These findings suggest that modulation of the endocannabinoid system may be a target for the development of possible therapeutic strategies for the treatment of this devastating cancer.

Cholangiocarcinomas are devastating cancers of intrahepatic and extrahepatic origin that are increasing in both their worldwide incidence and mortality rates (1, 2). The challenges posed by these often lethal biliary tract cancers are daunting; conventional treatment options are limited, and the only hope for long term survival is that of complete surgical resection of the tumor (1, 2). Conventional chemotherapy and radiation therapy are not effective in prolonging long term survival (1); therefore it is important to understand the cellular mechanisms of cholangiocarcinoma cell growth with a view to develop novel chemopreventive strategies.

Marijuana and its derivatives have been used in medicine for many centuries, and presently there is an emerging renaissance in the study of the therapeutic effects of cannabinoids. Ongoing research is determining that regulation of the endocannabinoid system may be effective in the treatment of pain (3, 4), glaucoma (5), and neurodegenerative disorders such as Parkinson disease (6) and multiple sclerosis (7). In addition, cannabinoids might be effective antitumoral agents because of their ability to inhibit the growth of various types of cancer cell lines in culture (8–11) and in laboratory animals (12).

The finding in the early 1990s of specific G-protein-coupled receptors for the psychoactive component of Cannabis sativa (–)−Δ⁹-tetrahydrocannabinol (13) led to the discovery of a whole endogenous signaling system now known as the endocannabinoid system (14). This system consists of the cannabinoid receptors (to date there are two, Cb12 and Cb2, as well as a putative involvement of the vanilloid receptor VR1), their endogenous ligands (endocannabinoids), and the proteins for their synthesis and inactivation (14). The cannabinoid receptors are seven-transmembrane domain proteins coupled to the Gi/o type of G-proteins (14). Cb1 receptors are found predominantly in the central nervous system but also in most peripheral tissues including immune cells, the reproductive system, and gastrointestinal tissues. In the central nervous system, Cb1 receptors are found predominantly on post-synaptic neuronal membranes. In peripheral tissues, Cb1 receptors are found predominantly on immune cells. The finding of the early 1990s of specific G-protein-coupled receptors for the psychoactive component of Cannabis sativa (–)−Δ⁹-tetrahydrocannabinol (13) led to the discovery of a whole endogenous signaling system now known as the endocannabinoid system (14). This system consists of the cannabinoid receptors (to date there are two, Cb12 and Cb2, as well as a putative involvement of the vanilloid receptor VR1), their endogenous ligands (endocannabinoids), and the proteins for their synthesis and inactivation (14). The cannabinoid receptors are seven-transmembrane domain proteins coupled to the Ga/o type of G-proteins (14). Cb1 receptors are found predominantly in the central nervous system but also in most peripheral tissues including immune cells, the reproductive system, and gastrointestinal tissues.

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2 The abbreviations used are: Cb1, cannabinoid receptor 1; Cb2, cannabinoid receptor 2; 2-AG, 2-arachidonylglycerol; AEA, anandamide; BrdUrd, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; dnFADD, dominant negative FADD; DR, death receptor; FADD, Fas-associated death domain protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; VR1, vanilloid receptor; Fasl, Fas ligand; MES, 4-morpholinolpropanesulfonic acid; shRNA, short hairpin RNA; BSA, bovine serum albumin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.
the gastrointestinal tract, and the lung (15–17). On the other hand, Cb2 receptors are found predominantly in the immune system, i.e. in tonsils, spleen, macrophages, and lymphocytes (15–17). To date, many endocannabinoids have been identified with varying affinities for the receptors (18). Anandamide (AEA) was the first endogenous ligand to be identified (15); it acts as a partial Cb1 agonist and weak Cb2 agonist. Another endocannabinoid is 2-arachidonylglycerol (2-AG), which activates both Cb1 and Cb2 receptors (19, 20). The chemical structures of these two endocannabinoids are shown in Fig. 1. Although the physiological role of the many other ligands has not yet been fully clarified, AEA and 2-AG have been implicated in a wide variety of physiological and pathological processes.

In addition to their cannabinoid receptor-mediated effects, endocannabinoids, and in particular AEA, are capable of mediating a plethora of receptor-independent cell signaling effects (21–26), most of which are via an interaction with cholesterol-rich microdomains of the cell membrane, also known as lipid rafts (21–23), and the synthesis of the raft-associated, sphingolipid moiety ceramide (24). These lipid rafts are thought to provide a platform for signaling molecules to concentrate and efficiently interact thereby facilitating the subsequent signal transduction process (27, 28). Indeed the subcellular localization of death receptors such as tumor necrosis factor (TNF) receptor 1, Fas, Fas-associated death domain protein (FADD), and other receptors of the TNF-related apoptosis-inducing ligand pathway are redistributed to lipid rafts in response to many apoptosis-inducing factors including a number of chemotherapeutic agents (29–32).

In the present study, we show the opposing effects of AEA and 2-AG on cholangiocarcinoma cell growth that were cannabinoid receptor-independent. In addition, we show that AEA mediated its inhibitory effects on cell growth by stimulating the accumulation of ceramide, stabilization of lipid rafts, and the recruitment of Fas and Fas ligand (FasL) to the lipid rafts. Conversely 2-AG increased cholangiocarcinoma cell growth and disrupted the organization of the lipid raft structures in the cell membrane.
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sion numbers: human Cb1, AY995204; Cb2, NM_001841; and VR1, NM_080704).

**Immunoblots**—Following trypsinization, H69, Mz-ChA-1, HuCC-T1, SG231, and HuH-28 cells (1 × 10⁶) were resuspended in lysis buffer as described previously (35, 39) and sonicated six times (30-s bursts). Immunoblots to detect Cb1, Cb2, and VR1 receptor expression were performed as described previously (35, 39).

**Immunofluorescence**—We detected Cb1, Cb2, and VR1 receptor expression in H69, Mz-ChA-1, HuCC-T1, HuH-28, and SG231 cells. Cells were seeded into 6-well dishes containing a sterile coverslip on the bottom of each well. Cells were allowed to adhere overnight, washed once in cold PBS, fixed to the coverslip with 4% paraformaldehyde (in 1 × PBS) at room temperature for 5 min, permeabilized in PBS containing 0.2% Triton X-100 (PBST), and blocked in 4% bovine serum albumin (BSA) in PBST for 1 h. Cb1, Cb2, and VR1 receptor antibodies were diluted (1:50 for Cb1 and VR1 antibodies and 1:100 for Cb2) in 1% BSA in PBST, added to the coverslips, and incubated overnight at 4 °C. Cells were washed 3 × 10 min in PBST, and a 1:50 dilution (in 1% BSA in PBST) of Cy3-conjugated secondary antibodies (Jackson Immunochemicals, West Grove, PA) was added for 2 h at room temperature. Cells were washed again and mounted into microscope slides with Prolong Antifade Gold containing DAPI and visualized using an Olympus IX-71 inverted confocal microscope.

**Cell Proliferation Assays**—Cholangiocarcinoma growth was evaluated by a 3-(4,5-dimethylthiazol-2-yil)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) proliferation assay in the Mz-ChA-1, HuCC-T1, HuH-28, and SG231 cell lines (40). After trypsinization, cells were seeded into 96-well plates (10,000 cells/well) in a final volume of 200 μl of medium. Cells were stimulated for 24, 48, and 72 h with AEA or 2-AG (1 nM to 10 μM). Cell proliferation was assessed using a colorimetric cell proliferation assay (CellTiter 96Aqueous, Promega Corp., Madison, WI), and absorbance was measured at 490 nm by a microplate spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA).

To determine possible mechanisms of action, Mz-ChA-1 cells were first preincubated for 1 h with various inhibitors prior to the addition of AEA or 2-AG for 48 h. The inhibitors utilized were SR141716 (a Cb1 antagonist, 10 nM (41)); SR144528 (a Cb2 antagonist, 10 nM (42)); AMG9810 (a VR1 antagonist, 10 nM (43)); pertussis toxin (G₁α protein inhibitor, 100 ng/ml (44)); methyl-β-cyclodextrin (0.1 mM), which depletes intracellular cholesterol and hence disrupts lipid rafts (45); filipin III (1 μg/ml), which binds and cross-links intracellular cholesterol and hence disrupts lipid rafts (46, 47); t-cycloserine (0.5 mM), a de novo ceramide synthesis inhibitor (48); or spiroepoxide (100 mM), which inhibits ceramide synthesis by hydrolysis of sphingomyelin (49).

In all cases, data were expressed as the fold-change of treated cells as compared with vehicle-treated controls. Statistical significance was determined using a t test, and p < 0.05 was considered significant.

Annexin V Labeling—Annexin V-labeling was used to evaluate the effects of AEA and 2-AG on cell cycle progression. Mz-ChA-1 cells were seeded in 6-well plates (500,000 cells/well) containing sterile coverslips on the bottom of each well and allowed to adhere overnight. Cells were stimulated with AEA (10 μM) or 2-AG (10 μM) for 24 h after which time coverslips were removed and rinsed in cold incubation buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) to remove excess media. Coverslips were then incubated in a solution containing Annexin V-biotin complex (1:50 dilution in incubation buffer) for 30 min at room temperature. After washing the coverslips to remove excess Annexin V-biotin, the cells were fixed in cold 4% paraformaldehyde (in PBS) for 10 min. Bound Annexin V-biotin complex was detected with Cy2-labeled streptavidin (1:200 dilution in PBS), mounted onto microscope slides with Prolong Antifade Gold containing DAPI, and visualized using an Olympus IX-71 inverted confocal microscope. The number of Annexin V-positive cells was counted and expressed as a percentage of total cells in five random fields for each treatment group. Data are the average ± S.E. of five fields in three independent experiments.

BrdUrd Labeling—BrdUrd labeling was used to evaluate the effects of AEA and 2-AG on cell cycle progression. Mz-ChA-1 cells were seeded in 6-well plates (500,000 cells/well) containing sterile coverslips on the bottom of each well and allowed to adhere overnight. Cells were stimulated with AEA (10 μM) or 2-AG (10 μM) for 48 h after which time BrdUrd (10 μM final concentration) was added to each well and incubated for a further 3 h at 37 °C. The coverslips were removed, rinsed in PBS to remove excess media, and fixed in 4% paraformaldehyde (in PBS) for 10 min at room temperature. DNA was denatured in 2 M HCl for 1 h at 37 °C and then neutralized with 0.5 M borate buffer (pH 9.5). The cell membranes were then permeabilized with PBST for 3 × 5 min. Nonspecific protein binding was then blocked with 4% BSA (in PBST) for 1 h at room temperature followed by a 2-h incubation with a 1:200 dilution (in 1% BSA in PBST) of anti-BrdUrd antibody. Coverslips were washed 3 × 5 min in PBST, and a 1:50 dilution (in 1% BSA in PBST) of Cy3-labeled anti-mouse secondary antibody was added and incubated for 2 h at room temperature. Excess antibody was removed by further washing in PBST, and the coverslips were mounted onto microscope slides with Prolong Antifade Gold containing DAPI, and visualized using an Olympus IX-71 inverted confocal microscope. The number of BrdUrd-positive nuclei was counted and expressed as a percentage of total cells in five random fields for each treatment group. Data are the average ± S.E. of five fields in three independent experiments.

Lipid Raft Labeling—Lipid rafts were visualized using the Vybrant® lipid raft labeling kit (Molecular Probes) with modifications. Briefly Mz-ChA-1 cells were seeded onto sterile coverslips placed in the bottom of 6-well culture dishes and allowed to adhere overnight. Cells were treated with vehicle, AEA, or 2-AG (both at 10 μM) for 4 h at 37 °C. Coverslips were then moved into new 6-well dishes for staining. Cells were incubated in the presence of 1 μg/ml (diluted in growth media) of fluorescent cholera toxin B subunit (Alexa Fluor 594 was the fluorochrome) for 15 min at 4 °C. This cholera toxin B subunit

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conjugate binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into lipid rafts (51, 52). After this incubation, the cells were gently washed with chilled PBS, fixed in 4% paraformaldehyde (in PBS) for 15 min at 4 °C, washed again in PBS, mounted onto microscope slides with Prolong Antifade Gold containing DAPI, and visualized using an Olympus IX-71 inverted confocal microscope. Analysis of the degree of co-localization between lipid rafts and Fas immunoreactivity was performed using a Pearson's correlation analysis that is part of the FluoView imaging software from Olympus. Using this analysis, complete co-localization of the two stains is represented by a Pearson's coefficient of 1.0, whereas complete non-colocalization gives a Pearson's coefficient of −1.0.

Isolation of Detergent-resistant Lipid Raft Fractions—Mz-ChA-1 cells (5 × 10⁶ cells) were seeded onto 10-cm cell culture plates and allowed to adhere overnight. Plates were then stimulated with vehicle, AEA (10 μM), 2-AG (10 μM), C6-ceramide (10 μM), spiroepoxide (100 nM), or spiroepoxide + AEA for 4 h (two plates for each treatment) after which time cells were scraped into ice-cold PBS, like treatments were pooled, and cells were pelleted by brief centrifugation. Cells were then lysed in 300 μl of 1× MES buffer (25 mM MES, 150 mM NaCl, 1 mM EDTA) containing 1% Triton X-100 as well as 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM aprotinin, and 1 mM sodium orthovanadate. The cell lysate was mixed with 600 μl of 60% OptiPrep (in 1× MES buffer). The resulting 40% OptiPrep solution was overlaid by a step gradient of 2.5 ml of 30% OptiPrep (in 1× MES buffer) and 1.0 ml of 5% OptiPrep and centrifuged at 20,000 × g for 20 h at 4 °C. Fractions were collected and analyzed by Western blotting as described previously (35, 39) using 20 μl of each fraction and specific antibodies against lyn, α-tubulin, flotillin-1, Fas, and FasL or β-actin.

Cholesterol Assays—Cholesterol content was determined in total cell extracts as well as in each of the lipid raft fractions (50-μl sample volume) after basal, AEA (10 μM), and

FIGURE 2. Expression of cannabinoid receptors CB1, CB2, and VR1 in cholangiocarcinoma cell lines as well as H69, SV-40-transformed normal cholangiocytes as shown by PCR (A), Western blotting (B), and immunofluorescence (C). Receptors were predominantly found in the cytoplasm, and no observable differences in receptor expression levels were seen between the cholangiocarcinoma cell lines and the H69 cell line. Cannabinoid receptor expression is shown in red with nuclei counterstained with DAPI (blue). Original magnification for immunofluorescence, ×60. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
2-AG (10 μM) treatment using the cholesterol determination kit from Molecular Probes according to the manufacturer’s instructions. For total cholesterol content, results were corrected for protein content in each sample and expressed as average ± S.E. in three samples. For the cholesterol content in lipid raft fractions, results were expressed as the average (± S.E.) of the percentage of the total cholesterol content in three samples.

Ceramide Labeling—Ceramide synthesis was measured using the immunofluorescence technique described previously with a specific anti-ceramide monoclonal antibody that in physiological in vitro and in vivo conditions is highly specific for ceramide and does not cross-react with sphingomyelin, cholesterol, or other phospholipids (53). Ceramide levels were visualized using an Olympus IX-71 inverted confocal microscope and quantitated using Adobe Photoshop where the number of ceramide-positive pixels (red) per nuclei (blue) was assessed. Data are the average ± S.E. in at least five random fields per treatment from three independent experiments.

Sphingomyelinase Assays—Sphingomyelinase activity was determined in samples 1, 2, and 4 h after AEA (10 μM) treatment using the sphingomyelinase assay kit purchased from Molecular Probes according to the instructions supplied by the vendor. This assay was performed under neutral and acidic conditions to assess both neutral and acidic sphingomyelinase activity. Data were expressed as -fold change in activity of four samples (average ± S.E.).

Transient Transfection—Mz-ChA-1 cells were seeded into 96-well plates (5,000 cells/well) and allowed to adhere overnight. Expression vectors containing dominant negative FADD or the control vector was transfected into the cells (0.1 μg of DNA/well) with 0.28 μl/well TransIT-LT1 transfection reagent (Mirus, Madison, WI) overnight at 37 °C. The cells were then incubated under basal conditions or stimulated with AEA (10 μM) for 48 h, and cell viability was determined by MTS assay as described above.

In addition, FADD expression was knocked down using shRNA plasmids containing a green fluorescent protein label.
from SuperArray. Four plasmids containing shRNA sequences against different regions of the human FADD gene were tested. Mz-ChA-1 cells were plated into 96-well plates and transiently transfected with the shRNA plasmids as described above. Transfection efficiency was monitored using fluorescence microscopy and determined to be equal in every well and at an efficiency of ~60%. MTS assays were then performed as described above. In a parallel experiment Mz-ChA-1 cells were seeded into 6-well plates and transfected with the shRNA plasmids. After 48 h, RNA was extracted as described above, and FADD expression was determined by real time PCR. Briefly reverse transcription was performed using the Reaction Ready first strand cDNA synthesis kit (SuperArray). The real time PCRs were set up in 96-well plates using the SYBR Green PCR Master Mix kit (SuperArray) and either specific primers for human FADD or glyceraldehyde-3-phosphate dehydrogenase (SuperArray). The fluorescence was monitored continuously throughout 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min) using the ABI Prism 7900HT sequence detection system. A ΔΔCT analysis was performed, and data were expressed as -fold change of FADD mRNA expression after correction for glyceraldehyde-3-phosphate dehydrogenase expression.

RESULTS

Cannabinoid Receptor Expression—All of the cholangiocarcinoma cell lines as well as the SV-40-transformed H69 cholangiocyte cell line expressed the RNA message (Fig. 2A) and protein (Fig. 2B) for the cannabinoid receptors Cb1, Cb2, and VR1. There was no obvious difference in levels of expression between the H69 cells and the carcinoma cell lines (Fig. 2, A and B). Immunofluorescence staining of these receptors showed a predominantly cytoplasmic expression in all cell lines consistent with previous studies (Fig. 2C) (54, 55).

Effect of Endocannabinoids on Cholangiocarcinoma Cell Growth—MTS proliferation assays revealed that AEA (10 μM) significantly decreased cell growth 48 h after stimulation (Fig. 3A) and up to 72 h (data not shown) in all cholangiocarcinoma cell lines studied. This concentration far exceeded the $K_i$ values for any of the cannabinoid receptors ($K_i = 89$ and 371 nM at Cb1 and Cb2, respectively (56, 57)). Concentrations below 10 μM had no effect on growth. In
contrast, the other most abundant endocannabinoid, 2-AG (10 μM), had a stimulatory effect on cholangiocarcinoma cell growth at 48 h (Fig. 3B) and 72 h (data not shown). Once again, there was no effect at concentrations below 10 μM.

The fact that these endocannabinoid-induced effects on cell growth were seen at concentrations that far exceed the binding affinities for any of the cannabinoid receptors suggests that these effects are probably receptor-independent. Indeed specific cannabinoid antagonists failed to block the AEA- and 2-AG mediated effects (Fig. 4, A and B). In addition, because these cannabinoid receptors are coupled to G_{i/o} proteins, we used pertussis toxin, an inhibitor of these proteins (44), to determine whether AEA or 2-AG is functioning through a G_{i/o} protein-coupled receptor. As seen in Fig. 4, A and B, pertussis toxin failed to inhibit either AEA-mediated inhibition or 2-AG stimulation of cholangiocarcinoma cell growth. These inhibitors, however, did effectively block the AEA-mediated suppression of forskolin-induced cAMP production in these cells (data not shown) in a manner similar to that in previous studies (58–60), indicating that the receptor antagonists are indeed functional at the concentrations used here. This indicates that neither endocannabinoid is exerting its effects through the cannabinoid receptors or any other G_{i/o}-coupled receptor.
Endocannabinoid-induced Effects on Apoptosis and Cell Cycle Progression—Because the MTS proliferation assay only gives information on changes in cell number and not the mechanism of any observed change (changes in cell death versus cell cycle progression), we evaluated the effects of AEA and 2-AG on apoptosis, as shown with Annexin V staining, and cell cycle progression, as indicated by BrdUrd incorporation. As seen in Fig. 5A, ~27% of the cholangiocarcinoma cells stained positive for Annexin-V under the basal conditions of our experiment. This value increased up to 60% after AEA treatment for 48 h, whereas 2-AG treatment decreased the incidence of apoptosis slightly but significantly down to 20% Annexin V-positive cells.

In contrast, during the 2 h of incubation in the presence of BrdUrd, ~40% of the cells took up BrdUrd into their DNA under the control conditions of these experiments (Fig. 5B). AEA treatment, however, decreased the amount of BrdUrd-positive cells and hence the rate of cell cycle progression, and 2-AG treatment increased the rate of cell cycle progression.

Endocannabinoids Exert Their Effects through Cholesterol-rich Lipid Rafts—Cannabinoids have been shown previously to have receptor-independent effects through interaction with lipid rafts (21–23). Therefore, we evaluated whether the receptor-independent effects of AEA and 2-AG shown here are also mediated through lipid rafts. Treatment of Mz-ChA-1 cells with the lipid raft disrupter methyl-β-cyclodextrin (45) had no effect on basal proliferation (Fig. 6A). However, pretreatment with methyl-β-cyclodextrin inhibited the AEA-mediated suppression of cell proliferation but failed to prevent the 2-AG-induced stimulation of proliferation (Fig. 6A). Similar results were observed following treatment of Mz-ChA-1 cells with filipin III, which disrupts lipid raft structure by a mechanism different from that of methyl-β-cyclodextrin (46) (Fig. 6B). Taken together these data suggest that AEA requires the integrity of the lipid raft membrane structure to be intact to induce the signal transduction pathways that ultimately result in increased apoptosis/cell cycle inhibition, whereas this membrane structure is not required for 2-AG-mediated increase in proliferation.

We then evaluated the effects of both AEA and 2-AG on the integrity of the lipid raft membrane structures by staining the GM1 gangliosides, which are an integral component of lipid rafts (51, 52). Fig. 7A, shows the lipid raft structures on the membranes of Mz-ChA-1 cells under basal conditions. The distribution and morphology of these lipid rafts did not appear to change significantly after AEA treatment. Stimulation of the cells with 2-AG, however, resulted in marked changes in the lipid raft staining patterns (Fig. 7A). The staining became more diffuse and apparently less membranous suggesting that perhaps 2-AG effectively disrupts the lipid raft structure and hence any lipid raft-mediated control of the intracellular machinery responsible for the initiation of apoptosis and/or cell cycle progression. To further demonstrate this phenomenon, we isolated the detergent-resistant
membrane fractions on an OptiPrep density gradient. Previous studies have shown that using a similar method the lipid rafts migrate to the interface between the 5 and 30% OptiPrep layers (61). Under the conditions used in the experiments outlined here, this corresponds to fractions 3 and 4. Using lyn and flotillin-1 as lipid raft markers, it can be seen that the lipid rafts did indeed migrate to the interface between the 5 and 30% OptiPrep layers under both basal conditions and after AEA treatment (i.e. lyn and flotillin-1 were found predominantly in fractions 3 and 4, Fig. 7B). However, 2-AG treatment resulted in the detection of lyn and flotillin-1 in many other fractions besides fractions 3 and 4 (Fig. 7B) suggesting that the integrity of the lipid rafts was compromised and that components that are usually found exclusively in the raft fraction diffused into the surrounding membrane. In these experiments, the non-raft-associated α-tubulin was used as a negative control to show that under our experimental conditions not all proteins are found in fractions 3 and 4 and that 2-AG does not disrupt the intracellular organization of all proteins (Fig. 7B).

**Neither AEA nor 2-AG Effects the Total Cholesterol Content, but 2-AG Effects the Distribution—** There was no observable difference in cholesterol content after 4 h of AEA or 2-AG treatment (Fig. 8). However, upon closer inspection, the distribution of membrane cholesterol in each of the membrane fractions used in Fig. 7B was markedly different after 2-AG treatment. In cells under basal conditions and AEA-treated cells, the majority of the cholesterol could be seen in fractions 3 and 4 corresponding to the lipid raft fractions, whereas after 2-AG treatment, less cholesterol was observed in fractions 3 and 4 and more was observed in the non-raft fractions 7 and 8 (Fig. 8). This once again suggests that 2-AG may be disrupting the lipid raft structures.

**AEA, but Not 2-AG, Increases Intracellular Ceramide Levels—** AEA has been shown previously to increase ceramide levels within the cell (24), and ceramide is an integral component of the lipid raft-mediated signaling cascade (62). For this reason we determined the effects of AEA and 2-AG on intracellular ceramide levels using a specific antibody. As can be seen in Fig. 9A, AEA increased the intracellular ceramide levels ~2-fold from basal conditions, whereas 2-AG had no effects. Furthermore both the acid and neutral sphingomyelinase activities were significantly induced from 1 to 4 h after AEA treatment (Fig. 9B). By treating Mz-ChA-1 cells with various concentrations of C2- and C6-ceramide, we showed that ceramide causes a decrease in cell proliferation similar to that seen after AEA treatment as shown by MTS assay (Fig. 9C). In addition, pretreatment of the cells with spiroepoxide and 1-cycloserine, inhibitors of the two pathways leading to the accumulation of ceramide, resulted in an inhibition of the AEA-mediated suppression of cell growth but no effect on the 2-AG-mediated enhancement of cell growth (Fig. 9D). Taken together, these data suggest that AEA, but not 2-AG, causes an increase in intracellular ceramide and that this ceramide accumulation is essential for the decrease in cellular proliferation seen after AEA treatment.

**Involvement of Fas Activation in AEA-mediated Proapoptotic Pathway—** Because AEA appears to activate proapoptotic events in cholangiocarcinoma cell lines, we wanted to determine what involvement the death receptor complex of the TNF superfamily has in this pathway. These death receptors are all capable of using FADD as a downstream effector molecule (63-65). Therefore, we transiently transfected a dnFADD expression vector into the Mz-ChA-1 cells to determine whether blocking the death receptor cascade has any effect on the AEA-induced cell death. Indeed transient dnFADD expression significantly inhibited the AEA-mediated suppression of cell growth as shown by MTS assay (Fig. 10A). In a similar experiment, we used shRNA vectors to knock down FADD expression. Transient transfection of the four different vectors knocked down the expression of FADD to 25–60% of the expression levels after transfection of the empty vector (Fig. 10B). This specific gene knockdown was enough to block the antiproliferative effects of AEA on Mz-ChA-1 cells. Total blockage of this antiproliferative effect by either dnFADD or the shRNA vectors was not achieved because the transfection efficiency was only 50–60%.

**FIGURE 6. Disruption of lipid rafts inhibits AEA- but not 2-AG-mediated effects on cholangiocarcinoma cell growth.** Mz-ChA-1 cells were pretreated with lipid raft disrupters 0.1 mM methyl-β-cyclodextrin (A) or 1 μg/ml filipin III (B) for 1 h prior to the addition of 10 μM AEA or 10 μM 2-AG. Cell proliferation was determined my MTS assays. Data are expressed as average ± S.E., and * denotes significance (p < 0.05) compared with basal treatment (n = 7).
In addition, using the lipid raft plasma membrane fractions, we showed that under basal conditions Fas and FasL were predominantly found in fractions 6 and 7, i.e. the non-lipid raft-containing fractions (Fig. 10C). Upon AEA stimulation these receptor components could also be detected in the lipid raft fractions (fractions 3 and 4; Fig. 10C), which presumably facilitates in the death receptor complex formation and subsequent activation of proapoptotic pathways. In addition, the recruitment of Fas and FasL into the lipid raft fractions was also observed after C6-ceramide treatment (Fig. 10C) suggesting that the accumulation of ceramide may indeed be responsible for the effects of AEA on lipid raft proteins. Furthermore pretreatment with the ceramide synthesis inhibitor spiroepoxide prior to the addition of AEA blocked the recruitment of both Fas and FasL into the lipid raft fractions.

In addition, using the lipid raft plasma membrane fractions, we showed that under basal conditions Fas and FasL were predominantly found in fractions 6 and 7, i.e. the non-lipid raft-containing fractions (Fig. 10C). Upon AEA stimulation these receptor components could also be detected in the lipid raft fractions (fractions 3 and 4; Fig. 10C), which presumably facilitates in the death receptor complex formation and subsequent activation of proapoptotic pathways. In addition, the recruitment of Fas and FasL into the lipid raft fractions was also observed after C6-ceramide treatment (Fig. 10C) suggesting that the accumulation of ceramide may indeed be responsible for the effects of AEA on lipid raft proteins. Furthermore pretreatment with the ceramide synthesis inhibitor spiroepoxide prior to the addition of AEA blocked the recruitment of both Fas and FasL into the lipid raft fractions.

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In support of these data, double staining experiments (Fig. 10D) showed that under basal conditions there was a moderate amount of overlap between the lipid raft staining (red) and Fas (green) indicated by the yellow color. Pearson’s correlation analysis of the degree of co-localization revealed a Pearson’s coefficient in the range of 0.48–0.65 in six random regions. However, after both AEA and C6-ceramide treatment, there was a greater degree of correlation between Fas immunoreactivity and lipid raft staining (Fig. 10D) with a Pearson’s coefficient in the range of 0.85–0.95. Other receptors in the TNF superfamily were not studied here; however, because they can also function in an FADD-dependent manner (63–65) and have been shown previously to be associated with lipid rafts (30, 32), their involvement in the AEA-mediated antiproliferative pathway cannot be ruled out.

DISCUSSION

The major findings of this study relate to the endocannabinoid system as a potential therapeutic target aimed to regulate cholangiocarcinoma cell growth. Using our different human cholangiocarcinoma cell lines we showed that 1) all cannabinoid receptors were expressed to a similar level in cholangiocarcinoma cells versus the SV-40-transformed H69 human cholangiocyte cell line, 2) the two major endocannabinoids, AEA and 2-AG, exert differential effects on cholangiocarcinoma cell growth via receptor-independent mechanisms, 3) AEA causes an antiproliferative and proapoptotic response through stabilization of lipid rafts and recruitment of Fas and FasL into the lipid rafts, and 4) the proliferative effect of 2-AG on cholangiocarcinoma cell growth may be due to the disruption of the lipid raft structure and hence disturbance of the subsequent lipid raft-dependent cell signaling cascades. These data suggest that therapies aimed at either modulating the relative levels of these two abundant endocannabinoids in vivo or mimicking the antiproliferative effects of AEA may prove beneficial in the treatment of cholangiocarcinoma.

Consistent with our observation that AEA has antiproliferative and proapoptotic properties, cannabinoids of various origins (endogenous, plant-derived, or synthetic analogues) have been shown to suppress cancer cell growth in vitro (8–11) as well as in vivo (12). Indeed recently a clinical pilot study to determine the safety of intracranial administration of (-)-Δ²-tetrahydrocannabinol in patients with glioblastoma demon-

expressed as a ratio of the pixel intensity of the red channel (ceramide) over the blue channel (DAPI) (average ± S.E. in five different fields from three independent experiments) * denotes significance (p < 0.05) from basal treatment. B, AEA treatment can increase both acid and neutral sphingomyelinase activity. Mz-CHA-1 cells were treated with AEA (10 μM) for various time points, and sphingomyelinase activity was assayed using a commercially available kit. Data are expressed as fold change in activity over basal activity (average ± S.E. of four samples). * denotes significance (p < 0.05). C, synthetic derivatives of ceramide can induce apoptosis in cholangiocarcinoma cell lines in a manner similar to that of AEA. Mz-CHA-1 cells were treated with various concentrations of C2- and C6-ceramide for 48 h. Cell proliferation was determined by MTS assay. Data are expressed as fold change from basal treatment (average ± S.E.; n = 7). * denotes significance (p < 0.05). D, inhibitors of ceramide synthesis block the antiproliferative effects of AEA. Mz-CHA-1 cells were pretreated for 1 h with 100 μM spiroepoxide or 0.5 mM L-cycloserine prior to the addition of 10 μM AEA or 10 μM 2-AG. Cell proliferation was determined by MTS assay. Data are expressed as fold change from basal treatment (average ± S.E.; n = 7). * denotes significance (p < 0.05).
A marked reduction in Ki67 staining within the tumor (66). This observation together with the very promising results observed in cell cultures and laboratory animals (8–12) suggest that administering cannabinoids alone or in conjunction with existing chemotherapeutic agents may be a promising treatment strategy for aggressive cancers of various origins. Another strategy may be to administer drugs that inhibit endocannabinoid uptake and subsequent degradation. Bifulco et al. (67) showed that administration of inhibitors of endocannabinoid cellular reuptake and endocannabinoid enzymatic hydrolysis into athymic mice with thyroid tumor xenografts effectively enhanced the endocannabinoid concentrations within the tumor and suppressed the tumor growth. In the present study, we also observed that, in contrast to AEA, 2-AG treatment resulted in an increase in cholangiocarcinoma cell growth. Although growth-promoting effects of cannabinoids in tumors of various origins have been demonstrated previously (26), to our knowledge, this is the first demonstration of the opposing effects of these two endocannabinoids within the one tumor type. To date, 2-AG is the only cannabinoid assayed that has this growth-promoting effect in cholangiocarcinoma cells. In light of this, as well as the fact that 2-AG is the most abundant endocannabinoid in tissues (14), caution must be taken when developing a chemotherapeutic strategy that results in an “across the board” increase in endocannabinoid levels. A treat-

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S. DeMorrow and G. Alpini, unpublished observation.
Endocannabinoids and Cholangiocarcinoma Growth

In the present study, we demonstrated that the effects of both AEA and 2-AG are mediated via cannabinoid receptor-independent mechanisms, consistent with previous observations (21–26). In fact, both growth-promoting and growth-suppressing actions of cannabinoids in a number of tumor types are receptor-independent and in the majority of cases were shown to be via interaction with lipid raft structures in the plasma membrane and/or regulation of ceramide levels (23–26, 68) in complete agreement with the data presented here. Many other anticancer drugs claim the plasma membrane as their site of action (69–71). However, little is known about the signaling mechanisms downstream from the plasma membrane that are involved in chemotherapy-induced cell death; this is an object of ongoing research in our laboratory.

In our studies, we demonstrated that 1) AEA induced an accumulation of ceramide within the cell, and 2) this ceramide accumulation was essential for the subsequent AEA-induced cell death. Ceramide is an integral component of the lipid raft structure and dramatically changes the biophysical properties of the rafts, allowing very tight packing of lipids within the rafts (72) and hence strongly stabilizing this membrane structure (73). This then facilitates the clustering or aggregation of various receptor molecules (74), which may theoretically have several functions including bringing many receptor molecules in close proximity to facilitate the transactivation between different receptor systems (74). It may also enhance the interaction between a receptor and its intracellular signaling molecules (75). In addition, raft ceramide may directly activate signal transduction pathways such as the Raf-1/MEK/ERK pathway (12, 76). The ceramide accumulation seen here after AEA treatment, we believe, is functioning to stabilize the lipid raft structure; however, the direct effect of ceramide on signal transduction pathways ultimately resulting in cell death cannot be ruled out and is a subject of our ongoing research. Indeed the tumor necrosis factor receptor molecule contains activator domains

FADD blocks the antiproliferative actions of AEA. Mz-ChA-1 cells were transfected with shRNA vectors or a control vector for 24 h. FADD expression was determined by real time PCR and expressed as relative FADD mRNA after correcting for changes in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Cell viability was determined by MTS assay after AEA treatment. MTS data are expressed as fold change from basal treatment (average ± S.E.; n = 14). * denotes significance from basal treatment (p < 0.05); # denotes significance from empty vector-transfected, AEA-treated samples (p < 0.05). Lane M, molecular size markers. C, detergent-resistant lipid rafts from Mz-ChA-1 cells were isolated on a discontinuous 5–40% Opti-Prep gradient. The lipid rafts elute in fractions 3 and 4, as indicated by the boxed area. Antibodies against Fas, Fasl, flotillin, and β-actin were used in immunoblotting to show the recruitment of these proteins into the lipid raft-containing fractions after AEA (10 µM), C2-ceramide (10 µM), spiroepoxide (100 nm), and spiroepoxide + AEA stimulation. D, co-localization of Fas immunoreactivity and lipid raft staining after AEA and C2-ceramide treatment. Mz-ChA-1 cells were treated with AEA (10 µM) or C2-ceramide (10 µM), and the lipid rafts were stained with Alexa Fluor 594-conjugated cholera toxin B subunit (red) as well as Fas immunoreactivity (green). Co-localization is indicated by yellow areas. Nuclei were counterstained with DAPI (blue). Under basal conditions, areas that are clearly Fas-positive but not lipid raft-positive or vice versa are indicated with the single arrowhead. After AEA and C2-ceramide treatment, areas that are both lipid raft-positive and Fas-positive are indicated with the double arrowhead. Scale bar, 10 µm.

**FIGURE 10.** Death receptors are required for the antiproliferative effects of AEA and are recruited into lipid rafts by AEA stimulation. A, overexpression of dnFADD blocks the antiproliferative actions of AEA. Mz-ChA-1 cells were transiently transfected with empty vector or an expression vector containing dnFADD for 24 h prior to the addition of 10 µM AEA. Cell proliferation was determined by MTS assay. Data are expressed as fold change from basal treatment (average ± S.E.; n = 14). * denotes significance from basal treatment (p < 0.05); # denotes significance from empty vector-transfected, AEA-treated samples (p < 0.05). B, transfection of shRNA vectors against human FADD blocks the antiproliferative actions of AEA. Mz-ChA-1 cells were transfected with shRNA vectors or a control vector for 24 h. FADD expression was determined by real time PCR and expressed as relative FADD mRNA after correcting for changes in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Cell viability was determined by MTS assay after AEA treatment. MTS data are expressed as fold change from basal treatment (average ± S.E.; n = 14). * denotes significance from basal treatment (p < 0.05); # denotes significance from empty vector-transfected, AEA-treated samples (p < 0.05). Lane M, molecular size markers. C, detergent-resistant lipid rafts from Mz-ChA-1 cells were isolated on a discontinuous 5–40% Opti-Prep gradient. The lipid rafts elute in fractions 3 and 4, as indicated by the boxed area. Antibodies against Fas, Fasl, flotillin, and β-actin were used in immunoblotting to show the recruitment of these proteins into the lipid raft-containing fractions after AEA (10 µM), C2-ceramide (10 µM), spiroepoxide (100 nm), and spiroepoxide + AEA stimulation. D, co-localization of Fas immunoreactivity and lipid raft staining after AEA and C2-ceramide treatment. Mz-ChA-1 cells were treated with AEA (10 µM) or C2-ceramide (10 µM), and the lipid rafts were stained with Alexa Fluor 594-conjugated cholera toxin B subunit (red) as well as Fas immunoreactivity (green). Co-localization is indicated by yellow areas. Nuclei were counterstained with DAPI (blue). Under basal conditions, areas that are clearly Fas-positive but not lipid raft-positive or vice versa are indicated with the single arrowhead. After AEA and C2-ceramide treatment, areas that are both lipid raft-positive and Fas-positive are indicated with the double arrowhead. Scale bar, 10 µm.
capable of inducing ceramide production by the activation of sphingomyelinase (77, 78), and this is FADD-dependent (79). Therefore it is also possible that instead of the AEA-induced accumulation of ceramide resulting in the stabilization of lipid rafts and recruitment of death receptors, AEA may first recruit the death receptor complex, which then results in the accumulation of ceramide. Regardless of the sequence of events, our data demonstrate a requirement for ceramide synthesis in the antiproliferative effects of AEA. The precise role of this phenomenon will be dissected further in our laboratory.

Data, such as those presented here, demonstrating the recruitment of protein components of the death receptor complexes into lipid raft structures are voluminous. It is widely accepted that both the TNF-receptor complex and the Fas-receptor complex are both recruited into lipid rafts in response to apoptosis-inducing drugs; this recruitment facilitates the receptor complex formation and is critical for their function (80–82). However, the stimulation of this recruitment into lipid rafts by cannabinoids has not been demonstrated previously in any cell type. In addition, these receptor complexes all require FADD to function (65). By overexpressing dominant negative FADD, we blocked the antiproliferative effects of AEA, suggesting that the formation of these death receptor complexes is required for the AEA-mediated effects on cholangiocarcinoma cell growth.

The effects of death receptor activation on cholangiocarcinoma cell growth have been demonstrated previously (83, 84). Tanaka et al. (83) showed that although the expression of TNF-related apoptosis-inducing ligand receptors (including DR4 and DR5) is not changed between resected cholangiocarcinoma samples and the surrounding normal liver tissue, exposure of cholangiocarcinoma cells to TNF-related apoptosis-inducing ligand (ligand of DR5) increased the incidence of apoptosis in vitro and in vivo and reduced the volume of a subcutaneous xenograft of human cholangiocarcinoma cells in vivo. In addition, Fas expression has been shown to negatively regulate cholangiocarcinoma cell growth in a xenograft model (84). Taken together, we propose that AEA facilitates the activation of these receptor complexes, in particular the Fas receptor, by their recruitment into lipid rafts, resulting in a proapoptotic phenomenon.

In conclusion, we have clearly demonstrated opposing actions of the endocannabinoids AEA and 2-AG on cholangiocarcinoma cell proliferation and have shown that these actions are via a cannabinoid receptor-independent but lipid raft-mediated pathway. Furthermore we have shown that the antiproliferative/proapoptotic actions of AEA are mediated via an accumulation of ceramide and the recruitment of the Fas death receptor into the lipid rafts. Cholangiocarcinoma has a very poor prognosis and survival rate; therefore we propose that the development of novel therapeutic strategies aimed at modulating the endocannabinoid system or mimicking the mode of action of AEA would prove beneficial for the treatment of this devastating disease.

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