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A Cannabinoid Quinone Inhibits Angiogenesis by Targeting Vascular Endothelial Cells

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ABSTRACT

Recent findings on the inhibition of angiogenesis and vascular endothelial cell proliferation by anthracycline antibiotics, which contain a quinone moiety, make this type of compound a very promising lead in cancer research/therapy. We have reported that a new cannabinoid anticancer quinone, cannabidiol hydroxyquinone (HU-331), is highly effective against tumor xenografts in nude mice. For evaluation of the antiangiogenic action of cannabinoid quinones, collagen-embedded rat aortic ring assay was used. The ability of cannabinoids to cause endothelial cell apoptosis was assayed by TUNEL staining and flow cytometry analysis. To examine the genes and pathways targeted by HU-331 in vascular endothelial cells, human cDNA microarrays and polymerase chain reaction were used. Immunostaining with anti-CD31 of tumors grown in nude mice served to indicate inhibition of tumor angiogenesis. HU-331 was found to be strongly antiangiogenic, significantly inhibiting angiogenesis at concentrations as low as 300 nM. HU-331 inhibited angiogenesis by directly inducing apoptosis of vascular endothelial cells without changing the expression of pro- and antiangiogenic cytokines and their receptors. A significant decrease in the total area occupied by vessels in HU-331-treated tumors was also observed. These data lead us to consider HU-331 to have high potential as a new antiangiogenic and anticancer drug.

Angiogenesis, the process of new blood vessel formation, is crucial for the development and progression of pathogenic processes of a variety of disorders, including tumor growth (Folkman, 1995). Much effort has been invested into the development of compounds with antiangiogenic activity. The findings on the ability of anthracycline antibiotics, a family of standard chemotherapy drugs that contain a quinone moiety, to inhibit angiogenesis and vascular endothelial cell proliferation, make this group of compounds very promising in cancer research/therapy (Gutman et al., 1994; Lennernas et al., 2003; Pastorino et al., 2003). Because anthracyclines are cardiotoxic, the development of quinonoid-based compounds that display antineoplastic and/or antiangiogenic activity but are less toxic than the standard anthracyclines is a major therapeutic goal. We have reported the synthesis of a new anticancer quinone, HU-331 (cannabidiol hydroxyquinone), from cannabidiol, one of the most abundant cannabinoids of *Cannabis sativa* (Kogan et al., 2004). HU-331 was found to be highly effective against tumor xenografts in *nude* mice (Kogan et al., 2004). Its mechanism of action is now under investigation in our laboratories.

The antiangiogenic properties of cannabinoids have not been thoroughly investigated so far. A possible antiangiogenic effect of cannabinoids is supported by the observation that these compounds modulate the response of cells to various growth factors (Bisogno et al., 1998; De Petrocellis et al., 1998; Melck et al., 1999, 2000; Di Marzo et al., 2001; Mimeault et al., 2003; Sanchez et al., 2003a,b; Hart et al., 2004), in

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; WIN-55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d*,*e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; HU-210, 11-hydroxy-Δ^8-tetrahydrocannabinol dimethyl heptyl; JWH-133, 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; ECGS, endothelial cell growth supplements; FGF, fibroblast growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; BAEC, bovine aortic endothelial cells; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; CBD, cannabidiol; VWF, von Willebrand factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VEGF, vascular endothelial growth factor.

particular vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), the central pro-angiogenic growth factors (Williams et al., 2003; Blázquez et al., 2004). Numerous cannabinoids that bind to CB_1 and/or CB_2 cannabinoid receptors (WIN-55,212-2, HU-210, JWH-133, and Δ^9-THC) inhibit vascular endothelial cell survival and migration (Blázquez et al., 2003). In addition, JWH-133 administration to glioma-bearing mice alters the expression of a number of genes related to the VEGF pathway (VEGF-A and -B, hypoxia-inducible factor 1-α, angiopoietin-2, Tie-1, and others) (Blázquez et al., 2004). The cannabinoid-induced inhibition of VEGF production was observed also in tumor cells from human glioblastoma multiforme biopsies (Blázquez et al., 2004). In skin carcinomas, cannabinoids target epidermal growth factor receptor, a growth factor receptor that plays a critical role in skin tumor angiogenesis (Casanova et al., 2002). Thus, cannabinoid administration reduces epidermal growth factor receptor activation (autophosphorylation) and mRNA levels in these tumors (Casanova et al., 2003). The expression of other proangiogenic factors, namely VEGF, placenta growth factor, and angiopoietin-2, was also strongly depressed by treatment of skin tumors with cannabinoids (Casanova et al., 2003). There are also some indications that cannabinoids might inhibit the metastatic spreading of tumor cells (Blázquez et al., 2003; Portella et al., 2003; Joseph et al., 2004).

This background prompted us to investigate the antiangiogenic action of quinonoid cannabinoid derivatives. We found that HU-331 is strongly antiangiogenic, both in vitro and in vivo, making this compound a promising scaffold for new antiangiogenic drugs.

Materials and Methods

Reagents and Cell Lines. HU-331 was synthesized as described previously (Kogan et al., 2004). Basic FGF, VEGF, endothelial cell growth supplements (ECGS), crystal violet and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma.

Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta and cultured in a moist atmosphere (5% CO_2) at 37°C in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), 50 μg/ml ECGS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins and cultured on gelatin-coated dishes in medium 199 supplemented with 10% FCS, 0.05% endothelial cell growth factor, and 100 μg/ml heparin. Cells were used between passages 2 and 6. The FL-1 human lung fibroblast cell line was cultured in DMEM supplemented with 2 mM L-glutamine, 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The HT-29 human colon carcinoma cell line was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS, 50 μg/ml ECGS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Cell Proliferation Test. Aliquots of cell suspensions were dispensed at 200-μl volumes into wells of 96-well tissue culture plates at a density of 0.02×10^6 cells/well. Various concentrations of cannabinoids were added, and their efficacy was tested 3 days after initiation of the cultures with the use of the MTT assay (Carmichael et al., 1987; Rubinstein et al., 1990). In each MTT assay, every concentration of the assayed substance was tested in five replicates. Assays were carried out in three different experiments. The inhibitory effect of the various compounds was calculated as percentage inhibition relative to cells treated with vehicle (0.5% ethanol).

TUNEL Staining and Flow Cytometry Analysis for Apoptosis Detection. Apoptosis was determined by TUNEL staining and by flow cytometry analysis of nuclear DNA content. For the former procedure, a TUNEL detection kit (Boehringer Mannheim, Mannheim, Germany) was used according to manufacturer's instructions; cells were cultured in coverslips, fixed in acetone for 10 min, and mounted with Mowiol mounting medium (Merck, Darmstadt, Germany) containing YOYO-1 iodide (1:1000; Molecular Probes, Leyden, The Netherlands) to stain cell nuclei (Blázquez et al., 2004). For the latter procedure, cell cultures were treated with trypsin-EDTA (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), collected by centrifugation, washed once, and incubated (1 h, room temperature) in PBS containing 1% (w/v) bovine serum albumin, 30% ethanol, and 5 μg/ml Hoechst 33342 (Molecular Probes). Fluorescence intensity was analyzed with a LSR flow cytometer (BD Biosciences, San Jose, CA). Ten thousand cells were recorded in each determination.

Ex Vivo Rat Aortic Ring Assay. Rat tail collagen was prepared as described by Burbridge and West (2001). Tendons were removed from fresh rat tails and washed once in 70% ethanol and then 10 times in sterile PBS. Then they were dried overnight under UV light and weighed. Acetic acid (0.01%) was added (300 ml per 1 g of tendons), and the tendons were stirred for 1 week at 4°C. The solution was then centrifuged, and the clear supernatant containing the collagen was used. The final collagen solution was obtained by mixing 7.5 volumes of collagen, 1 volume of 10× minimal essential medium, and 1.5 volumes of $NaHCO_3$, pH adjusted to 7.4 with NaOH. Thoracic aortas were removed from 250-g male Sabra rats and immediately transferred to culture dishes containing serum-free Bio-MPM medium (Biological Industries Ltd., Kibbutz Beit Haemek, Israel). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors; special care was taken to not damage the aortic wall. One millimeter aortic rings were sectioned and extensively rinsed in five consecutive washes of sterile Bio-MPM medium. Twenty-four-well tissue culture grade plates were covered with 300 μl of collagen and allowed to gel for 20 min at the room temperature. Ring-shaped explants of aorta were then placed (with lumen parallel to plate bottom) on the collagen-coated wells and covered with an additional 200 μl of collagen and allowed to gel for additional 15 min at room temperature. Then, 2 ml of Bio-MPM medium containing VEGF/FGF (25 ng/ml) as positive controls and VEGF/FGF + the tested substance was added (in triplicate). The plates were incubated in a moist atmosphere (5% CO_2) at 37°C for 1 week; then the medium was removed and formalin was added for an overnight fixation. The rings were then stained with crystal violet, photographed, and analyzed using the Image-Pro software (Media Cybernetics, Inc., Silver Spring, MD). Mean values were computed from triplicate images. Four parameters were chosen to quantify the antiangiogenic effects of HU compounds: 1) the area of endothelial cells that proliferate from aorta, 2) the number of new vessels, 3) the average vessel length, and 4) the maximal vessel length.

In Vivo Experiments. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies. Tumors were grafted into 15 *nude* mice by s.c. flank inoculation of 0.2×10^6 HT-29 cells in RPMI 1640 medium without FCS. The animals were assigned randomly to three groups of five and injected i.p. from day 2 after cell injection with vehicle (1:1:18 ethanol/Emulphor/PBS), 5 mg/kg of HU-331 (three times a week, 15 mg/kg/week), or 0.83 mg/kg (three times a week, 2.5 mg/kg/week) doxorubicin. The concentrations of HU-331 and doxorubicin were the maximal possible concentrations chosen based on a preliminary toxicity study. At day 40, the mice were euthanized and the tumors were fixed in 4% buffered formaline and then paraffin-embedded. The sections were deparaffinized and rehydrated, and samples were treated as described previously (Blázquez et al., 2004). For immunodetection of blood vessels, sections were incubated (1.5 h, room temperature) with anti-CD31 antibody (1:400; Pharmingen, San Diego, CA). After washing with PBS, slices were further incubated (1 h, room temperature, darkness) with the secondary goat anti-mouse antibody Alexa Fluor 594 (1:500; Molecular Probes). After washing with PBS, sections were mounted with Mowiol mounting medium (Merck) containing

YOYO-1 iodide (1:1000; Molecular Probes) to stain cell nuclei. Fluorescence images were acquired using an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany). Morphometric analysis of the vasculature was performed with Metamorph-Offline software (Universal Imaging, Downingtown, PA).

cDNA Arrays. HUVECs were treated with vehicle or HU-331 for 12 h. Total RNA was extracted with TRIzol (Invitrogen, Barcelona, Spain) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of 50 μCi of [α-^{33}P]dATP. Purified radiolabeled probes from two different experiments were hybridized to human-cDNA Endothelial Cell Biology Microarray membranes (GEArray Q Series; Superarray Bioscience Corporation, Frederick, MD) according to manufacturer's instructions (see http://www.superarray.com for a detailed list of the genes analyzed). Hybridization signals were detected by a Cyclone Storage Phosphor System (PerkinElmer Life and Analytical Sciences, Boston, MA) and analyzed by Phoretix array software (Nonlinear Dynamics Inc., Newcastle-upon-Tyne, UK); redundant housekeeping genes were used in the blots as internal controls for normalization. The selection criteria were set conservatively throughout the process, and the genes selected were required to exhibit at least a 2-fold change of expression in each of the two experiments performed and a p value <0.01.

Real-Time Quantitative PCR. Real-time quantitative PCR was performed by using LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, IN) and Light Cycler detector as described previously (Alvarez et al., 2004). Each sample was assayed in triplicate. Quantitative expression values were extrapolated from separate standard curves, and normalized to β-actin. Specific oligonucleotide primers used are shown in Table 1. Reverse-transcription PCR determinations were carried out in parallel.

Statistics. Results shown represent mean ± S.E.M. Statistical analysis was performed by analysis of variance with a post hoc analysis by the Student-Neuman-Keuls test or by unpaired Student's t test.

Results

Cannabinoids Inhibit Vascular Endothelial Cell Proliferation. The structures of the cannabinoids assayed are presented in Fig. 1A. All these compounds were able to inhibit BAEC growth as determined by the MTT test. As shown in Fig. 1B, the quinonoid derivatives were more active than their parent compounds, and HU-331 was the most potent one. The effect of the latter compound (4.8 μM, 24- or 72-h incubation) was independent of cell density (at least 10,000 to 30,000 cells/cm^2; data not shown) and much more remarkable in BAEC than in FL-1 fibroblasts (Fig. 1C). Endocannabinoids (anandamide, noladin ether, 2-arachidonoylglycerol) were tested as well, but they showed little or no effect on BAEC proliferation (data not shown).

TABLE 1
Oligonucleotide primers

MCP1	
Forward	5′-TCGCTCAGCCAGATGCAATC-3′
Reverse	5′-CTTGGGTTGTGGAGTGAGTG-3′
MMP1	
Forward	5′-AAATCCTGTCCAGCCCATCG-3′
Reverse	5′-GCTTCAAGCCCATTTGGCAG-3′
COX2	
Forward	5′-AATTGCTGGCAGGGTTGCTG-3′
Reverse	5′-CTACCAGAAGGGCAGGATAC-3′
VWF	
Forward	5′-GAGCCTGAGTGCAACGACAT-3′
Reverse	5′-GAGCATTTGCACTCCATGGC-3′
OPG	
Forward	5′-CCTGTGTGAGGAGGCATTCT-3′
Reverse	5′-TACGAAGCTGCTCGAAGGTG-3′
PLA2	
Forward	5′-GGCATGGAGTGACACATACG-3′
Reverse	5′-GAAGAACAGGAAGGCCAGGT-3′
β-actin	
Forward	5′-GAGCGGGAAATCGTGCGTGACATT-3′
Reverse	5′-GAAGGTAGTTTCGTGGATGCC-3′

HU-331 Induces Apoptosis of Vascular Endothelial Cells. We next examined whether HU-331 directly affects the viability of vascular endothelial cells. Analysis of HU-331-treated BAEC by flow cytometry shows that HU-331 induces apoptosis of BAEC, as determined by counting of hypodiploid cells (Fig. 2, A and B). This was supported by TUNEL staining experiments (Fig. 2C). HUVEC also underwent HU-331-mediated apoptosis (Fig. 2D), although they tended to be slightly less sensitive than BAEC to HU-331 action (percentage of cell viability relative to vehicle-treated cells after incubation for 24 h with 4.8 μM HU-331: HUVEC, 41 ± 10; BAEC, 32 ± 8; n = 4, not significant).

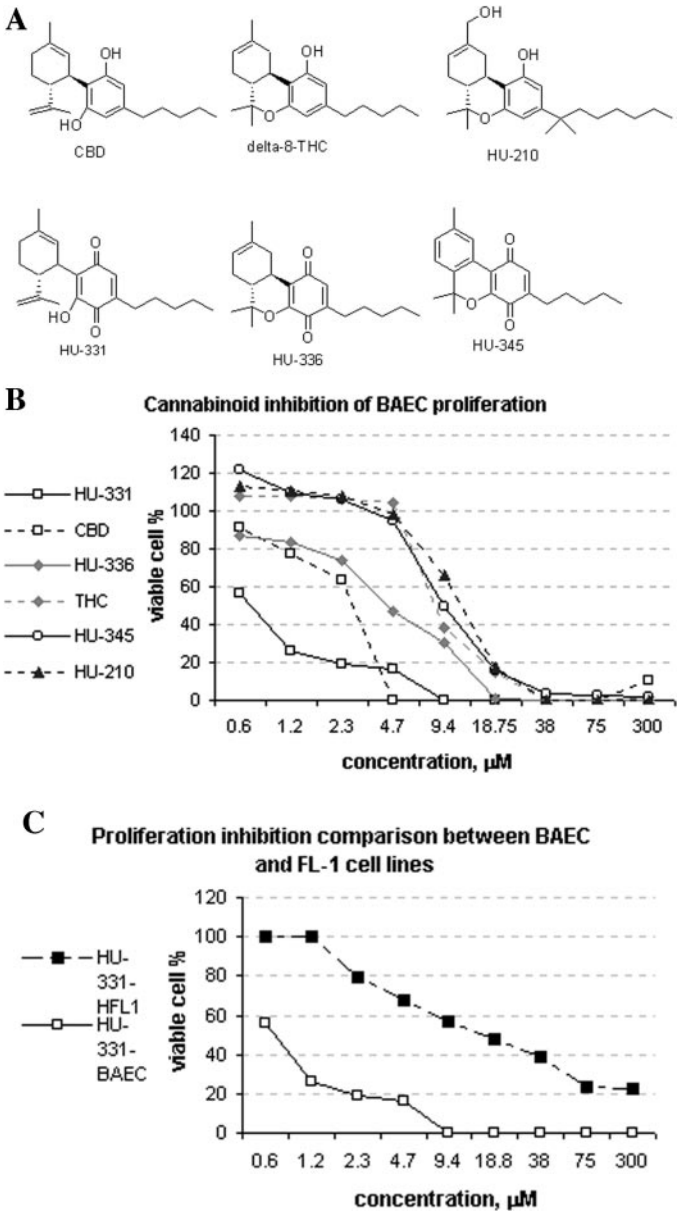

Fig. 1. Cannabinoids inhibit BAEC proliferation. A, cannabinoid structures. B, cannabinoid inhibition of BAEC proliferation. C, inhibition of BAEC versus FL-1 fibroblast cell proliferation by HU-331.

HU-331 and Other Cannabinoid Quinones Inhibit Angiogenesis in a Rat Aortic Ring Model. The ex vivo aortic ring assay was employed for the evaluation of cannabinoids on angiogenesis. This semiquantitative ex vivo assay system is usually considered to come closest to simulating the in vivo situation, not only because it includes the surrounding nonendothelial cells but also because the endothelial cells have not been preselected by passaging and thus are not in a proliferative state at the time of explantation.

Collagen-embedded aortic rings were incubated for 5 to 7 days with HU-331, in the presence of FGF or VEGF (or with FGF/VEGF alone for the positive control). HU-331 was strongly antiangiogenic, and even at 300 nM, it was able to inhibit aortic ring angiogenesis. All the parameters determined (area of endothelial cells that proliferate from aorta, number of new vessels formed, average vessel length, and maximal vessel length) were affected by HU-331. We decided to stop at a concentration of 300 nM; although this concentration caused a prominent effect in some of the parameters measured (such as vessel length), it had no statistically significant effect on the others (area occupied by proliferated cells). In control aortas treated with FGF, there was lower new vessel formation than in those treated with VEGF, but there were more proliferated cells. Upon HU-331 treatment, the number of new vessels formed was not only lower, but even those that were formed were shorter and the area occupied by proliferated cells was smaller (Figs. 3 and 4).

Other cannabinoid quinones, HU-336 (tetrahydrocannabinol quinone) and HU-345 (cannabinol quinone), were able to inhibit aortic ring angiogenesis more potently than their parent compounds (THC and cannabinol, respectively) or CBD, but were less potent than HU-331, which is the most effective cannabinoid quinone in our series, and a clear dose-response relationship could not always be observed for these compounds (data not shown). The major plant cannabinoids, CBD and THC, also possess some antiangiogenic activity. Both CBD and THC have been shown to be essentially nontoxic (Rozenkrantz et al., 1981). CBD and THC showed much

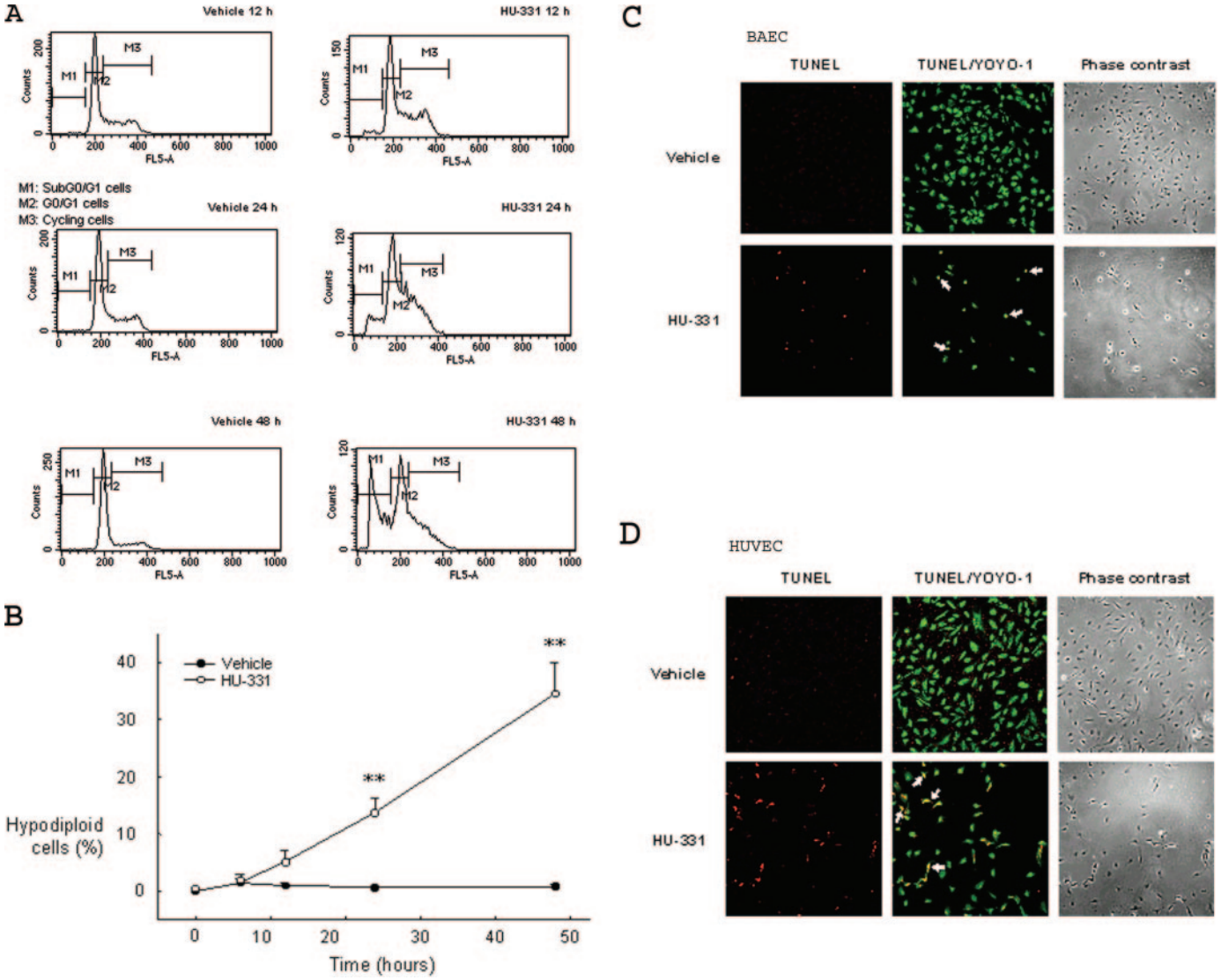

Fig. 2. HU-331 induces apoptosis of vascular endothelial cells. A and B, BAEC were cultured for the periods indicated with vehicle or 4.8 μM HU-331 and flow cytometry analysis of nuclear DNA content was performed. Representative cell cycle profiles are shown in A. Hypodiploid cell counting from five different experiments is shown in B. Significantly different (**, $p < 0.01$) from vehicle-treated cells. C and D, TUNEL staining of BAEC (C) and HUVEC (D) after treatment with vehicle or 4.8 μM HU-331 for 18 h. Examples of TUNEL-positive cells are pointed with arrows.

less activity than the quinonoid derivatives in this assay. Thus at 2.3 μM, there was no inhibition of angiogenesis (data not shown). Both CBD and THC had some biphasic effects: at very low concentrations (~50 nM), they induced some aortic ring angiogenesis; CBD caused proliferation but not new vessel formation, whereas THC induced new vessel formation but not proliferation. Cannabinol had slight pro-angiogenic activity at almost all concentrations tested, and HU-210 had nearly the same potency as CBD (data not shown).

HU-331 Inhibits Tumor Angiogenesis. We have previously shown that HU-331 inhibits the growth of tumors generated by subcutaneous inoculation of HT-29 human colon cancer cells in mice. Thus, in HU-331-treated mice, the mean tumor area was more than twice as small as that in control animals (Kogan et al., 2004). We therefore analyzed whether HU-331, besides affecting physiological angiogenesis, also impairs tumor angiogenesis. Immunostaining of CD31, a marker of endothelial cells, followed by morphometric analyses revealed a significant decrease in the total area occupied by vessels in HU-331-treated tumors (Fig. 5, A and B). This was due to a lower vascular density (number of blood vessels per area unit; Fig. 5C) rather than to changes in blood vessel size (area per vessel; Fig. 5D). In contrast, doxorubicin, another antitumoral quinone, was unable to significantly affect tumor vascularization (percentage of section area occupied by vessels: vehicle-treated mice, 4.3 $\pm$ 0.5; doxorubicin-treated mice, 5.5 $\pm$ 0.6; HU-331-treated mice, 2.6 $\pm$ 0.4), supporting the idea that HU-331 antiangiogenic action is structurally specific.

HU-331 Affects the Expression of Vascular Endothelial Cell Genes. To examine the genes and pathways targeted by HU-331 in vascular endothelial cells, we used commercially available human cDNA microarrays. For this purpose, cells were incubated with vehicle or HU-331 for 12 h to detect early changes in gene expression that could determine the further apoptotic outcome. A total of 96 genes associated with the major functions of endothelial cells—including angiogenesis, vascular tone, and cell activation, adhesion, and injury—were analyzed, of which 81 were considered to be expressed in reliable amounts. Using stringent gene selection criteria (see *Materials and Methods*), we found that HU-331 alters the expression of six genes related to various basic functions of vascular endothelial cells (Fig. 6, A and B). Thus, HU-331 increased the mRNA levels of matrix metalloproteinase-1, cyclooxygenase-2, and osteoprotegerin, whereas it decreased the mRNA levels of monocyte chemotactic protein-1, von Willebrand factor (VWF), and cytosolic phospholipase A2. The observed changes in these six genes were confirmed by real-time quantitative PCR (Fig. 6C) and by reverse-transcription PCR (Fig. 6D). No significant effect of HU-331 was observed on a number of ubiquitous modulators of angiogenesis, such as the proangiogenic cytokines VEGF, FGF-2, angiopoietin 1 (Ang-1), and angiopoietin 2 (Ang-2); the VEGF receptors VEGFR-1, VEGFR-2, and VEGFR-3 and the angiopoietin receptor Tie-2; and the antiangiogenic cytokines thrombospondin-1, endostatin, angiostatin, and vasostatin (Fig. 6E). Likewise, HU-331 did not affect the expression of various vascular endothelial cell adhesion molecules such as integrins α5, αV, β1, and β3; selectins E, L, and P; intercellular adhesion molecules 1, 2, and 3; cadherin 5; and occludin (data not shown).

Discussion

Herein, we provide evidence that angiogenesis is potently inhibited by the cannabinoid derivative HU-331. In particular, this compound induces vascular endothelial cell apoptosis without affecting the expression of the most prominent cytokines and receptors involved in the control of angiogenesis; it blunts not only basal angiogenesis but also tumor angiogenesis. The most parsimonious interpretation of these findings is that HU-331 inhibits angiogenesis by directly inducing apoptosis of vascular endothelial cells upstream of expression of pro/antiangiogenic cytokines and their receptors. Nevertheless, it may not be ruled out that HU-331 interferes with intracellular pathways modulated by pro/antiangiogenic cytokines, as has been shown previously for cannabinoids that bind to CB receptors (Blázquez et al., 2004).

Cannabinoids that bind to CB receptors have been re-

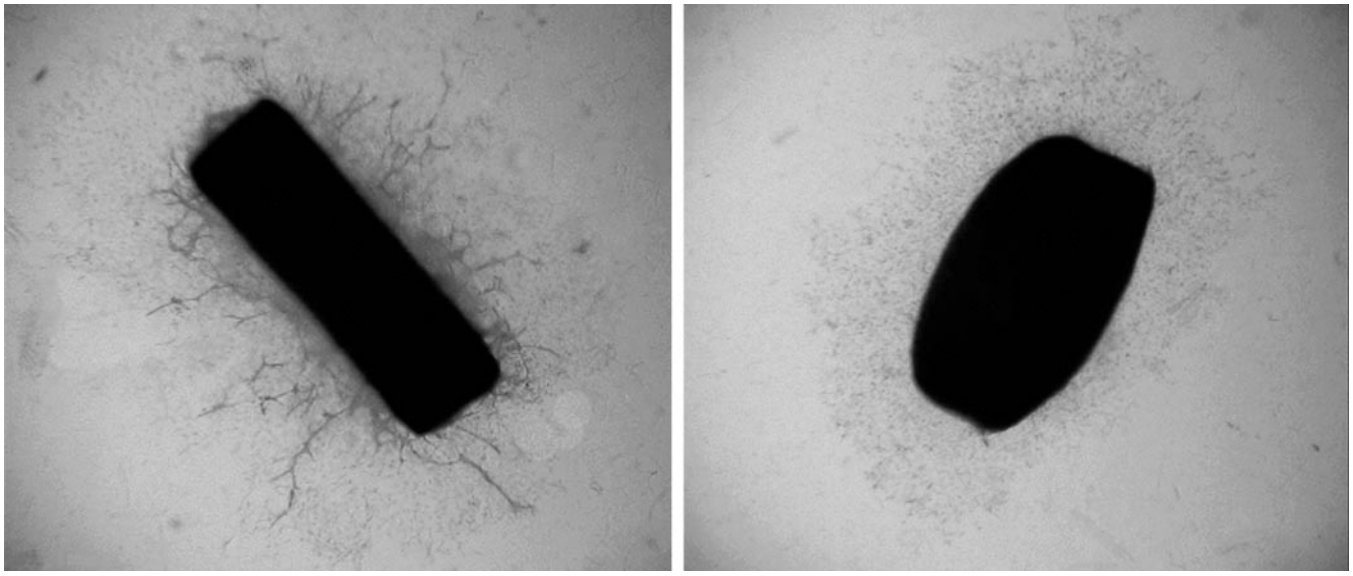

Fig. 3. Example of HU-331 action on rat aortic ring angiogenesis. Left, rat aortic ring exposed to VEGF. Right, rat aortic ring exposed to VEGF and 600 nM HU-331.

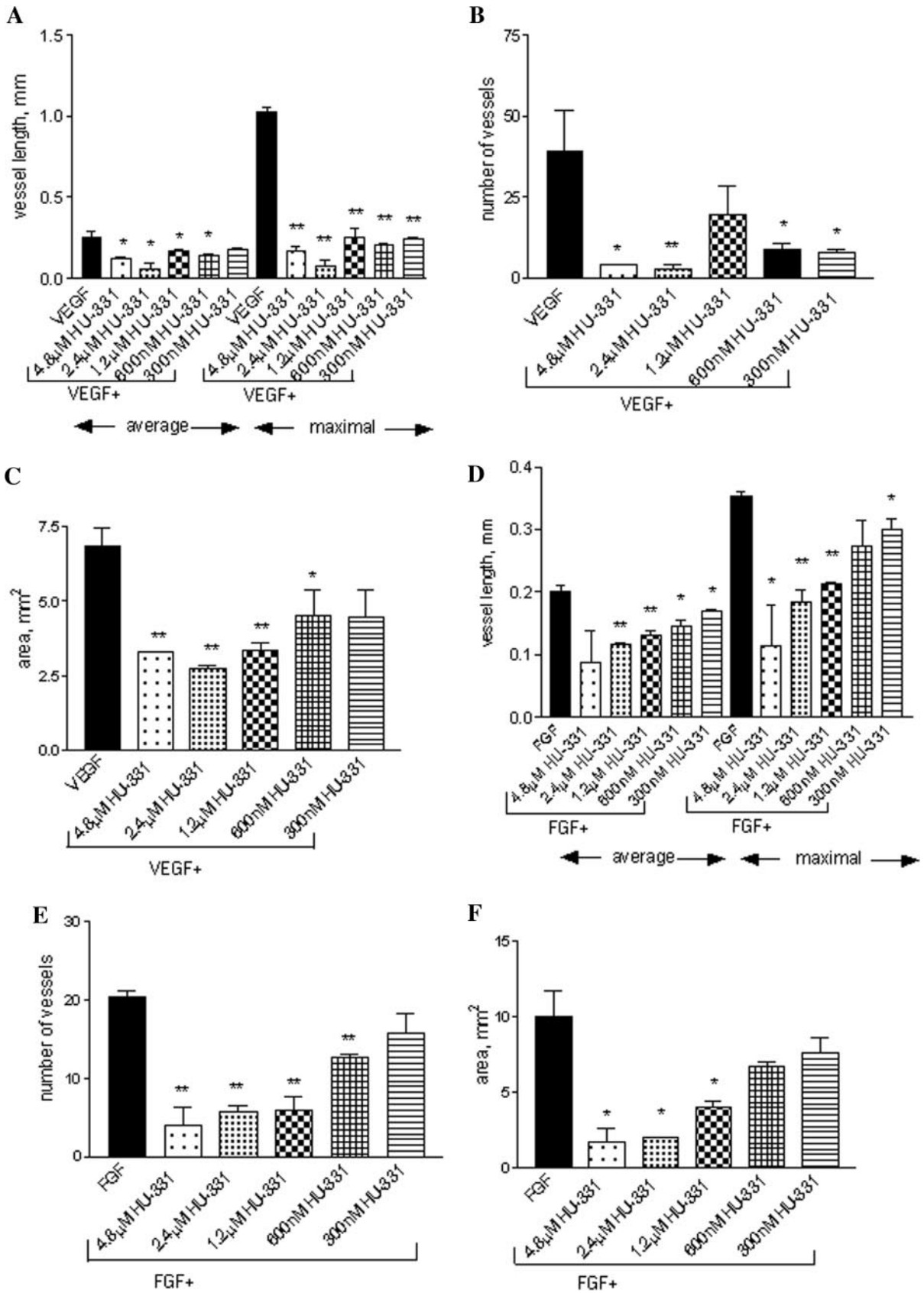

Fig. 4. HU-331 interferes with rat aortic ring angiogenesis. A, influence of HU-331 on average and maximal VEGF-mediated-new-formed vessel length. B, influence of HU-331 on the number of VEGF-mediated new-formed vessels. C, influence of HU-331 on the area occupied by VEGF-mediated proliferated aortic cells. Significantly different (*, $p < 0.05$; **, $p < 0.01$) from VEGF-treated aorta. D, influence of HU-331 on average and maximal FGF-mediated-new-formed vessel length. E, influence of HU-331 on the number of FGF-mediated new-formed vessels. F, influence of HU-331 on the area occupied by FGF-mediated proliferated aortic cells. Significantly different (*, $p < 0.05$; **, $p < 0.01$) from FGF-treated aorta.

ported to inhibit tumor angiogenesis. Thus, immunohistochemical analyses of mouse models of glioma (Blázquez et al., 2003) and skin carcinoma (Casanova et al., 2003) have shown that cannabinoid administration turns the vascular hyperplasia typical of actively growing tumors to a pattern of blood vessels characterized by small and differentiated capillaries. This is associated with a reduced expression of VEGF and other proangiogenic cytokines (Blázquez et al., 2003; Casanova et al., 2003; Portella et al., 2003) as well as of the VEGF receptors VEGFR-1 (Portella et al., 2003) and VEGFR-2 (Blázquez et al., 2004). Vascular endothelial cells express various functional receptors for cannabinoids, including the CB_1 receptor (Liu et al., 2000), the CB_2 receptor (Blázquez et al., 2003), the tentative abnormal cannabidiol receptor (Járai et al., 1999), and the TRPV1 vanilloid receptor (Golech et al., 2004), which control important cell functions such as migration (Blázquez et al., 2003; Mo et al., 2004), survival (Blázquez et al., 2003), and vascular tone (Wagner et al., 1997; Bátkai et al., 2001). It is possible, however, that CB receptor agonists and HU-331 use different primary cellular targets to induce their antiangiogenic actions. Thus, CB receptor agonists seem to act prominently in tumor xenografts, most likely via inhibition of tumor cell proliferation and survival (Guzmán, 2003) and impairment of VEGF production and signaling in tumor cells (Portella et al., 2003; Blázquez et al., 2004). HU-331 affects the expression of a large number of genes involved in very primary vascular endothelial cell functions. They include the genes encoding the matrix proteinase matrix metalloproteinase-1, the eicosanoid-generating enzyme cyclooxygenase-2, the bone growth/remodeling factor osteoprotegerin, the C-C chemokine monocyte chemotactic protein-1, the prothrombotic protein VWF, and the arachidonic acid-generating enzyme phospholipase A2. These observations suggest a broad-based impact of HU-331 in vascular endothelial cell biology.

The use of cannabinoids in medicine is severely limited by their psychoactive effects. As the unwanted psychotropic effects of cannabinoids are mediated largely or entirely by the CB_1 receptor, a conceivable possibility would be to use cannabinoids that do not target that receptor, such as cannabi-

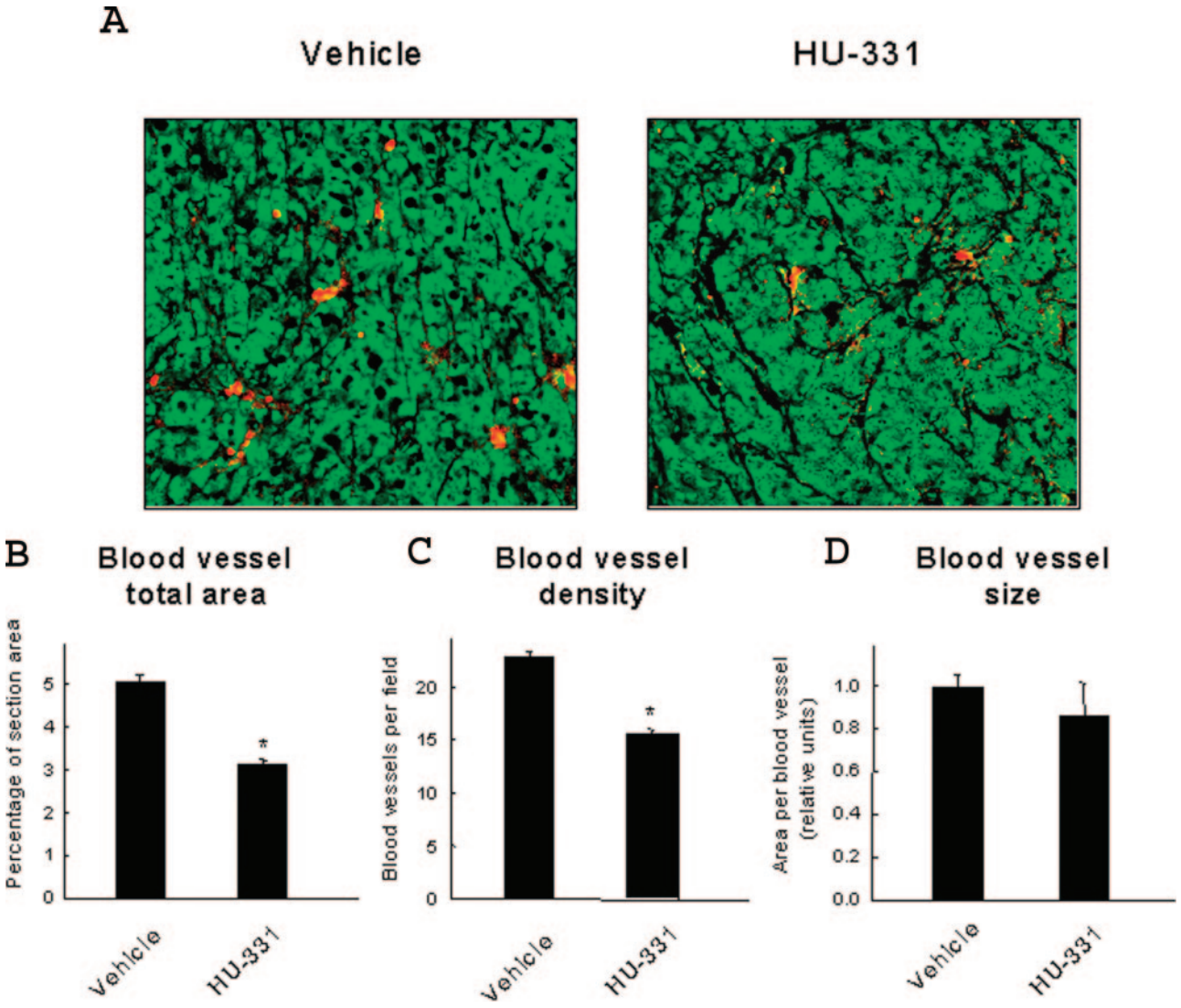

Fig. 5. HU-331 inhibits tumor angiogenesis. HT-29 cells were injected subcutaneously in mice and tumors were generated. Animals were treated with either vehicle or HU-331 (5 mg/kg, i.p.) for 35 days, tumors were immunostained for CD31, and morphometric analysis of tumor vasculature was performed (B–D). A representative section of CD31 labeling is shown in A (CD31, red; cell nuclei, green). Significantly different (*, $p < 0.05$) from vehicle-treated mice.

diol (Mechoulam and Shvo, 1963; Pertwee et al., 2005) or its more potent derivative HU-331 (Kogan et al., 2004). The present observations open the possibility of finding psychoactivity-devoid therapeutic strategies for the management of angiogenesis-related diseases. The precise pharmacodynamic profile of HU-331 remains, however, to be established.

Agents representing virtually every class of chemotherapeutic drugs have been reported to have antiangiogenic activity on the basis of some in vitro or in vivo assays (Miller et al., 2001). However, many of them are antiangiogenic only at near-full cytotoxic concentrations, and hence the clinical relevance of these effects is not obvious. Differential cytotoxicity

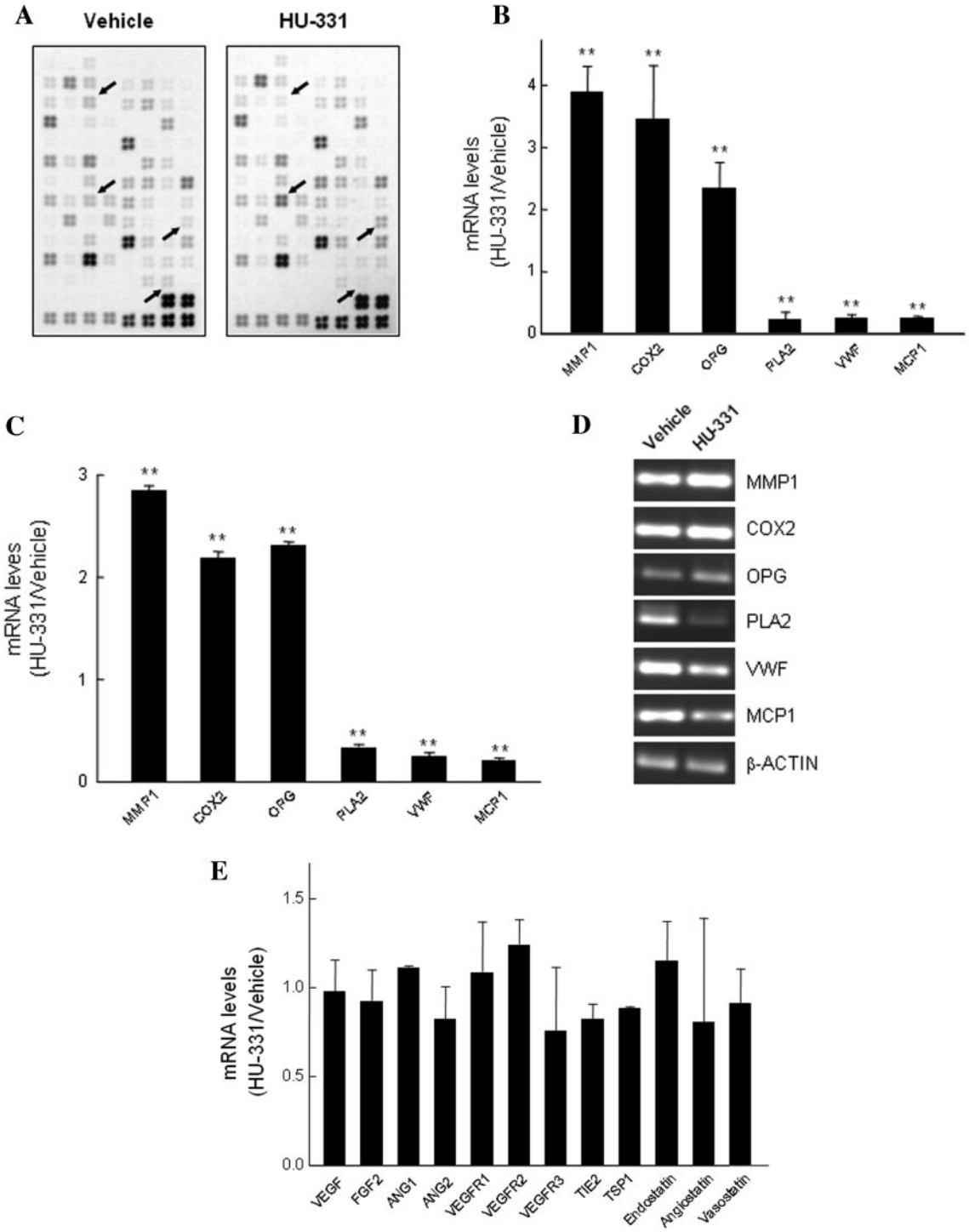

Fig. 6. Changes in HUVEC gene expression profile after HU-331 treatment. HUVEC were incubated with either vehicle or 4.8 μM HU-331 for 12 h. A and B, equal amounts of RNA obtained from vehicle- and HU-331-treated cells were hybridized to endothelial cell biology cDNA array membranes. Two identical experiments were performed. A, examples of affected genes in experiment 1 are pointed with arrows. From top to bottom: MCP1, MMP1, COX2, and VWF. B, genes significantly affected by HU-331. See text for abbreviations and further details. C and D, validation of the changes in gene expression observed in the cDNA array experiments as determined by real-time quantitative PCR (C) and reverse-transcription PCR (D). E, lack of effect of HU-331 on angiogenesis-related genes in the cDNA array experiments. Significantly different (**, $p < 0.01$) from vehicle-treated cells.

seems to be a useful discriminator of true antiangiogenic activity from simple toxicity. In our work with HU-331, this novel anticancer compound was found to inhibit both endothelial cell proliferation in vitro and angiogenesis in ex vivo rat aortic rings assay at concentrations lower than those required for its effect on most human cancer cell lines (Kogan et al., 2004). Its antiangiogenic effect is evident also in vivo in tumor xenografts, in which it reduces the vascular count (present report) as well as tumor size (Kogan et al., 2004) at doses well tolerated by mice. The antiangiogenic activity of a compound may be mediated through its indirect effects on cancer cells, for example by inhibiting the production of proangiogenic factors by tumor cells. However, this does not seem to be the case in our study, because HU-331 and other quinonoid cannabinoids are antiangiogenic in ex vivo aortic ring assay, in which the proangiogenic growth factors (VEGF and FGF) are added exogenously to the medium. Thus, the antiangiogenic effect of HU-331 is probably mediated by its direct activity on endothelial cells.

Regarding the antiangiogenic action of the classic cannabinoids, the antiangiogenic properties of these compounds are of interest but have been little investigated. Cannabidiol is the most active plant cannabinoid. Although cannabidiol does not bind to CB_1 and CB_2 cannabinoid receptors, a new vascular target for it has been discovered (Begg et al., 2005). Cannabidiol action on angiogenesis is less potent than that of HU-331.

Taken together, our data shows that both the plant cannabinoid cannabidiol and its quinonoid derivative HU-331 are able to inhibit angiogenesis. In particular, HU-331 possesses a very potent antiangiogenic activity. Because this compound has anticancer properties and is more selective and potent in our assays than numerous known chemotherapeutic drugs, it may have a high potential as a new antiangiogenic drug. The antiangiogenic effects of other cannabinoids, especially CBD, are also of interest, because CBD is nontoxic even at doses of 50 mg/kg and, being nonpsychotropic, it can be administered in high doses.

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