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Tumor-promoting effects of cannabinoid receptor type 1 in human melanoma cells

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ABSTRACT

The role of endocannabinoid system in melanoma development and progression is actually not fully understood. This study was aimed at clarifying whether cannabinoid-type 1 (CB1) receptor may function as tumor-promoting or -suppressing signal in human cutaneous melanoma. CB1 receptor expression was measured in human melanoma cell lines by real-time PCR. A genetic deletion of CB1 receptors in selected melanoma cells was carried out by using three different short hairpin RNAs (shRNAs). Performance of target gene silencing was verified by real-time PCR and Western blot. The effects of CB1 receptor silencing on cell growth, clonogenicity, migration capability, cell cycle progression, and activation of mitogenic signals was tested. Lentiviral shRNAs vectors targeting different regions of the human CB1 gene led to a significant reduction in CB1 receptor mRNA and a near complete loss of CB1 receptor protein, compared to control vector (LV-c). The number of viable cells, the colony-forming ability and cell migration were significantly reduced in cells transduced with CB1 lentiviral shRNAs compared to LV-c. Cell cycle analyses showed arrest at G1/S phase. p-Akt and p-ERK expression were decreased in transduced versus control cells. Findings of this study suggest that CB1 receptor might function as tumor-promoting signal in human cutaneous melanoma.

1. Introduction

The endocannabinoid system is a ubiquitous complex signaling network with many biological activities both in the central and peripheral tissues (Hillard, 2015; Maccarrone et al., 2015; Minocci et al., 2011; Nieri et al., 2003a, b). It is composed by endogenous ligands (anandamide and 2-arachidonoyglycerol), cannabinoid receptors type-1 (CB1) and type-2 (CB2), enzymes involved in endocannabinoid biosynthesis and metabolism, and more recently discovered members (Di Marzo, 2009).

Several studies have demonstrated that CB1 and CB2 receptor stimulation can block proliferation, motility, invasion, adhesion and induce apoptosis in various types of cancer, both *in vitro* and *in vivo* (Chakravarti et al., 2014; Pisanti et al., 2013). Nonetheless, the role of the endocannabinoid system in cancer pathophysiology appears to be controversial since several lines of evidence showed that it can be iper-activated in cancer (Velasco et al., 2015). With regard to this, much attention has been recently devoted to study the role of the endocannabinoid signaling in skin biology (Maccarrone et al., 2015). For example, it has been reported that genetic ablation of CB1 and CB2 receptors protected from ultraviolet light-induced skin carcinogenesis (Zheng et al., 2008). In apparent contrast with these findings are those obtained in *in vivo* models of melanoma showing that cannabinoids administration could inhibit tumor growth (Blázquez et al., 2006) and such an effect occurred in wild-type mice but not in those lacking CB1 and CB2 receptors (Glodde et al., 2015). Moreover, three studies carried out on the same melanoma cell line showed that CB1 selective agonists, mixed CB1/CB2 agonists and CB1 antagonists can decrease cell proliferation (Adinolfi et al., 2013; Blázquez et al., 2006; Carpi et al., 2015).

The current study was aimed at investigating whether genetic deletion of CB1 receptor by short hairpin RNAs (shRNAs) may affect proliferation, clonogenicity and cell migration in well-characterized human melanoma cell lines. Understanding the precise role of CB1 receptor may provide further insights into molecular pathogenesis of human melanoma thus offering new potential therapeutic targets for innovative pharmacological strategies.

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2. Materials and methods

2.1. Cell culture

The human A375, MeWo and 501 Mel melanoma cells and the human embryonic kidney 293 cells (HEK-293T) were obtained from the American Type Culture Collection (ATCC). Melanoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, Milan, Italy). Normal human epidermal melanocytes (NHEM) (PromoCell GmbH, Germany) were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Melanocyte Growth Medium M2 (PromoCell GmbH, Germany). SSM2c cultures were obtained from the patient metastatic melanoma tissue after approved protocols by the Ethics Committee and were obtained as previously reported (Santini et al., 2014). HEK-293T cells were grown as specified (Santini et al., 2014). Cell morphology was examined under light microscopy.

2.2. Lentiviral vector production and transduction

The lentiviral vectors used were pLKO.1-puro (LV-c) (Sigma-Aldrich, Milan, Italy), as a control, pLKO.1-puro-shCB1-1 (LV-shCB1-1) to target the coding region of CB1 receptor, and pLKO.1-puro-shCB1-2 (LV-shCB1-2) and pLKO.1-puro-shCB1-3 (LV-shCB1-3) to target the CB1 3′-untranslated region. The sequences were CCGGAGCTCATTAAGACGGTGTTTGCTCGAGCAAACACCGTCTTAATGAGCTTTTTTG for LV-shCB1-1, CCGGTGCTAATGTTTCCATAGTTTACTCGAGTAAACTATGGAAACATTAGCATTTTTG for LV-shCB1-2 and CCGGTAGTATCAGAGATGTCCATTTCTCGAGAAATGGACATCTCTGATACTATTTTTG for LV-shCB1-3. All vectors were packaged in HEK-293T cells and produced as described previously (Stecca and Ruiz i Altaba, 2009). shCB1 stable cell lines were established by transducing A375 and 501Mel cells with purified virus. Puromycin, at 2 and 0.5 μg/ml, was used to select stable transduced pools of A375 and 501Mel cells, respectively.

2.3. Real-time PCR

Total RNA was isolated and retrotranscribed, as previously reported (Carpi et al., 2014). Quantitative PCR amplifications were carried out using TaqMan probe for CB1 receptors and 18S (Thermo Fisher Scientific, MA, USA) on MiniOpticon CFX 48 real-time PCR Detection System (Bio-Rad). Data were analyzed by δ-Ct method using β-actin and 18-S as housekeeping genes. Real-time PCR was performed using SsoFast Eva Green Supermix (Ref. 172–5201; Bio-Rad, California, USA). Samples were amplified using the following thermal profile: 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 15 s, followed by annealing for 30 s and 72 °C for 30 s, with a final step at 65 °C for 5 s. β-Actin primer sequences were 5′-AACTGGAACGGTGAAGGTGAC-3′ (F) and 5′-GACTTCCTGTAACAACGCATCTC-3′ (R) and the annealing temperatures (Ta) was 61 °C.

2.4. Western blot analysis

The expression of CB1 receptor was evaluated in melanoma cell lysates by Western blot experiments as previously described (Carpi et al., 2014). Briefly, 30 μg of protein was diluted with Laemmli sample buffer 2×, boiled for 8 min at 96 °C and separated by 10% sodium dodecyl sulphate-polyacrylamide electrophoresis gels. Proteins were transferred to nitrocellulose membranes by electroblotting at 4 °C. To avoid non-specific immunodetection, membranes were incubated for 45 min in T-TBS (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 8) containing 5% non-fat milk. Blots were then incubated overnight at 4 °C with a rabbit anti-CB1 antibody (1:500 dilution, PA1-745; Thermo Fischer), anti-ERK1 (p44)/ERK2 (p42) (1:200 dilution, Ref. sc-514302, Total ERK, Santa Cruz Biotechnology), anti-phosphotyrosine 204-ERK1(p44)/ERK2(p42) (1:500 dilution, Ref. sc-7383, Santa Cruz Biotechnology), anti-p-Akt1-2-3 (1:500 dilution, sc-7985-R Santa Cruz Biotechnology) and with a mouse anti-β-actin (1:5000 dilution; Merck-Millipore) diluted in T-TBS containing 1% non-fat milk. After extensive washes in T-TBS, immunoreactive bands were detected by incubation with horseradish peroxidase-conjugated secondary anti-bodies anti-rabbit (Merck-Millipore, # MAB201P) and anti-mouse (Sigma-Aldrich, A4416), respectively. Signals were revealed by chemiluminescent detection (ImageQuant LAS 4000, GE Healthcare) and densitometric analysis of bands was carried out with the ImageJ64 software (http://rsb.info.nih.gov/ij/download.html).

2.5. Cell growth assay

Growth curves were carried out by seeding 3×10^3-transduced cells per well on 12-well plates. Cell growth was assessed at 3, 5 and 7 days after seeding. Cells were stained by trypan blue dye (Sigma-Aldrich, Milan, Italy) and counted with Burker's camera.

2.6. Cell colony forming assay

Melanoma transduced cells were seeded at low density (500 cells/well) in triplicate and grown for 10 days in normal serum medium. Colonies of melanoma cells stably transduced with LV-c and LV-shCB1 were washed with PBS twice, fixed with methanol for 15 min, and stained with 0.05% crystal violet for 15 min at room temperature. Colonies containing >50 individual cells were counted under light microscopy. Pictures were taken at a 4× magnification.

2.7. Cell cycle assay

Cells (2×10^5) were seeded on 6-well plates, incubated in medium with 1% FBS for 24 h and then with 10% FBS for 24 h. To determine cell cycle distribution, cells were centrifuged for 5 min at 5000 rpm. Cells were resuspended in 500 μl of propidium iodide solution (trisodium citrate 0.1% w/v, NP40 0.1% w/v, PI 50 μg/ml) (Merck Millipore, Calbiochem, #537059), incubated for 30 min at 4 °C in darkness and subjected to flow cytometry analysis. Flow cytometry was performed by using a FACSCanto II (BD Bioscience). The acquired FACS data were analyzed by ModFit LT software (Verity Software House, Inc.) to determine the percentage of the population in the G0/G1, S and G2/M phase.

2.8. Cell migration assay

An IBIDI culture insert (IBIDI GmbH) consisting of two reservoirs separated by a 500 μm thick wall was used. An equal number of control and CB1-silenced melanoma cells (70 μl; 3×10^5 cells/ml) were added into the two reservoirs of the same insert and incubated at 37 °C/5% CO_2. After 24 h, the insert was gently removed creating a gap of 500 μm. Dishes were washed twice with PBS to remove the detached cells, and incubated using the complete growth medium. Wound closure (cells migrating into the scratched empty space) was monitored by light microscopy. Pictures were taken at a 4× magnification after 12 h. The percentage of scratch area was calculated using WimScratch program (Wimasis S.L., Córdoba, Spain).

2.9. Statistic analysis

Data were presented as mean ± standard error of the mean (SEM) from at least three independent experiments. Statistical analysis was performed by one-way or two-way ANOVA followed by Dunnetts's or Bonferroni's multiple comparison test. p values less then 0.05 were considered as statistically significant.