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Pharmacological inhibition of MAGL attenuates experimental colon carcinogenesis

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ABSTRACT

Colorectal cancer (CRC) is a major health problem in Western countries. The endocannabinoid 2-arachidonoyl-glycerol (2-AG) exerts antiproliferative actions in a number of tumoral cell lines, including CRC cells. Monoacylglycerol lipase (MAGL), a serine hydrolase that inactivates 2-AG, is highly expressed in aggressive human cancer cells. Here, we investigated the role of MAGL in experimental colon carcinogenesis. The role of MAGL was assessed *in vivo* by using the xenograft and the azoxymethane models of colon carcinogenesis; MAGL expression was evaluated by RT-PCR and immunohistochemistry; 2-AG levels were measured by liquid chromatography mass spectrometry; angiogenesis was evaluated in tumor tissues [by microvessel counting and by investigating the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) proteins] as well as in human umbilical vein endothelial cells (HUVEC); cyclin D1 was evaluated by RT-PCR. MAGL and 2-AG were strongly expressed in tumor tissues. The MAGL inhibitor URB602 reduced xenograft tumor volume, this effect being associated to down-regulation of VEGF and FGF-2, reduction in the number of vessels and down-regulation of cyclin D1. In HUVEC, URB602 exerted a direct antiangiogenic effect by inhibiting FGF-2 induced proliferation and migration, and by modulating pro/anti-angiogenic agents. In experiments aiming at investigating the role of MAGL in chemoprevention, URB602 attenuated azoxymethane-induced preneoplastic lesions, polyps and tumors. MAGL, possibly through modulation of angiogenesis, plays a pivotal role in experimental colon carcinogenesis. Pharmacological inhibition of MAGL could represent an innovative therapeutic approach to reduce colorectal tumor progression.

Abbreviations: 2-AG, 2-arachidonoyl glycerol; ACF, aberrant crypt foci; AEA, anandamide; AOM, azoxymethane; BSA, bovine serum albumin; HCT116, colon adenocarcinoma cell line; CRC, colorectal cancer; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's medium; EGM-2, endothelial growth medium-2; FAAH, fatty acid amide hydrolase; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; FBS, foetal bovine serum; HUVEC, human umbilical vein endothelial cell line; i.p., intraperitoneally; MAGL, monoacylglycerol lipase; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PBS, phosphate buffer solution; TBS, tris-buffered saline; VEGF, vascular endothelial growth factor.

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1. Introduction

Colorectal cancer (CRC) is the most prevalent gastrointestinal malignancy worldwide [1,2]. It has been estimated that, in 2015, it will be the third most common cause of cancer-related deaths in the USA with a prediction of 132,700 new diagnosed cases [3]. It has been known that sporadic CRC develops, in the majority of cases, as a consequence of the sequential accumulation of genetic alterations and epigenetic modulations [4,5]. The disease begins with the generation of pre-neoplastic lesions, known as aberrant crypt foci (ACF), and, through the formation of polyps, develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer [6]. The microenvironment plays a crucial role in the tumor growth and a considerable number of key transduction signals are involved in tumor microenvironment progression, such as angiogenesis and cycle progression. In particular, it is largely reported that angiogenesis is important for the development of solid tumors because the dissemination of tumors requires new blood vessel growth [7,8]. Despite clear progress in physiopathology and cure of colon cancer, current therapeutic interventions may fail to prevent disease progression to metastatic dissemination. Therefore, it is crucial to identify different molecular actors playing a key role in CRC oncogenesis.

Monoacylglycerol lipase (MAGL) is a serine hydrolase that converts long chain monoacylglicerols to glycerol and fatty acid. The enzyme is localized in different areas of brain and in peripheral tissues, including the gastrointestinal tract [9–11]. MAGL is upregulated in aggressive human ovarian, prostate, breast cancer cells and in primary tumors, where it promotes migration, invasion, survival, and *in vivo* tumor growth [12,13].

MAGL plays a predominant role in catalyzing the hydrolysis of the endocannabinoid 2-arachidonoyl glycerol (2-AG) [14–16], whereas other enzymes are more involved in the hydrolysis of the endocannabinoid anandamide and related acylethanolamides (*i.e.* palmitoylethanolamide and oleoylethanolamide) [17,18]. 2-AG is generated "*on demand*" through *stimulus*-dependent cleavage of membrane phospholipid precursors and its levels are regulated by the balance between its production and degradation [19]. This endocannabinoid is involved in a variety of physiological and physiopathological processes. Relevant to this study, 2-AG has been shown to exert antiproliferative effects in a number of cancer cell lines [20–22], including colorectal cancer cells [23]; conversely, reducing endogenous 2-AG-levels increases cell invasion [24]. Importantly, the increase of endogenous 2-AG levels as a consequence of MAGL inhibition was shown to reduce prostate cancer invasion *in vitro* [25].

Our understanding of how MAGL can impact on cancer progression is so far hindered by under-reported and controversial data [26,27], since it has been suggested that the enzyme may mediate both cancerogenic [28] and anti-cancerogenic effects [29]. Furthermore, the effect and role of MAGL in relation to 2-AG levels and angiogenesis have been not evaluated to date. Therefore, in the present study we have investigated the role of MAGL in colon carcinogenesis by evaluating the potential chemopreventive and curative effect of the selective MAGL inhibitor URB602. Specifically, the effects of URB602 on tumor progression, 2-AG levels and angiogenesis were evaluated.

2. Materials and methods

2.1. Drugs and reagents

Azoxymethane (AOM) was purchased from Sigma (Milan, Italy). URB602 and Matrigel™ were obtained from Cayman Chemical (Cabru SAS, Arcore, Italy) and BD Biosciences (Buccinasco, Milan, Italy), respectively. All reagents for cell cultures were obtained from Sigma, Bio-Rad Laboratories (Milan, Italy) and Microtech Srl (Naples, Italy). The vehicles used for *in vivo* (10% ethanol, 10% Tween-20, 80% saline, 2 ml/kg) and *in vitro* (0.1% DMSO) experiments had no effect on the responses under study.

2.2. Cell culture

The human colon adenocarcinoma cell line (HCT116, ATCC from LGC Standards, Milan, Italy) and the human umbilical vein endothelial cell line (HUVEC, Promocell, Heidelberg, Germany) were used. Cells were routinely maintained in 75 cm^2 polystyrene flasks, at 37 °C in a 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium (DMEM) for HCT116 or in endothelial growth medium (EGM-2) for HUVEC. DMEM was supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 2 mM L-glutamine. EGM-2, containing VEGF, R^3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000 (Clonetics, Cambrex Bio Science Walkersville, USA) was supplemented with 10% FBS. Cells were used at passage 20–27 for HCT 116 and 1–10 for HUVEC. The medium was changed every 48 h in conformity with the manufacturer's protocols.

2.3. Animals

Male ICR mice (weighting 25–30 g) and athymic nude female 4-weeks old mice were purchased from Harlan Italy (S. Pietro al Natisone, UD, Italy). All mice were used after 1 week-acclimation period (temperature 23 ± 2 °C; humidity 60%, free access to water and food). Athymic female mice, fed *ad libitum* with sterile mouse food, were maintained under pathogen-free conditions. The experimental protocol was evaluated and approved by the Institutional Animal Ethics Committee for the use of experimental animals and conformed to guidelines for the safe use and care of experimental animals in accordance with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council (86/609/ECC and 2010/63/UE).

2.4. Colorectal cancer xenograft model

HCT 116 cells (2.5×10^6) were injected subcutaneously into the right flank of each athymic mouse for a total volume of 200 μl [50% cell suspension in phosphate buffer solution (PBS) and 50% Matrigel™]. Ten days after inoculation (once tumors had reached a size of 250–300 mm^3), mice were randomly assigned to control and treated groups, and treatments were initiated. Tumor size was measured every day by digital caliper, and tumor volume was calculated according to the modified formula for ellipsoid volume (volume = $\pi/6 \times$ length $\times$ width^2). Mice were euthanized when the endpoint tumor volume was of 2000 mm^3. The MAGL inhibitor URB602 [30], at the dose of 5 mg/kg, was given intraperitoneally (i.p.) every day for all the duration of the experiment. URB602 dose was selected on the basis of previous published work which showed selective inhibitory effects of URB602 on MAGL enzyme without psychoactive effects [31]. Xenograft tumor tissue as well as healthy tissue were collected and analyzed after 8 days of treatment. Healthy tissue derived from contralateral flank (*i.e.* the flank not injected with xenografted cells) which includes skin, muscle, connective tissue and nerves.

2.5. Quantitative (real-time) RT-PCR analysis

Healthy tissues and tumors collected from xenografted mice were immediately immersed into RNA Later and stored at −20 °C until analysis. HCT 116 and tissues were homogenized in 1.0 ml of TRIzol (Invitrogen) following the manufacturer's instructions and