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Activated kRas protects colon cancer cells from cucurbitacin-induced apoptosis: The role of p53 and p21

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ABSTRACT

Cucurbitacins have been shown to inhibit proliferation in a variety of cancer cell lines. The aim of this study was to determine their biological activity in colon cancer cell lines that do not harbor activated STAT3, the key target of cucurbitacin. In order to establish the role of activated kRas in the responsiveness of cells to cucurbitacins, we performed experiments in isogenic colon cancer cell lines, HCT116 and Hke-3, which differ only by the presence of an activated kRas allele. We compared the activity of 23, 24-dihydrocucurbitacin B (DHCB) and cucurbitacin R (CCR), two cucurbitacins that we recently isolated, with cucurbitacin I (CCI), a cucurbitacin with established antitumorigenic activity. We showed that cucurbitacins induced dramatic changes in the cytoskeleton (collapse of actin and bundling of tubulin microfilaments), inhibited proliferation and finally induced apoptosis of both HCT116 and Hke-3 cells. However, the presence of oncogenic kRas significantly decreased the sensitivity of cells to the three cucurbitacins tested, CCR, DHCB and CCI. We confirmed that mutational activation of kRas protects cells from cucurbitacin-induced apoptosis using nontransformed intestinal epithelial cells with inducible expression of kRasV12. Cucurbitacins induced the expression of p53 and p21 predominantly in HCT116 cells that harbor mutant Ras. Using HCT116 cells with targeted deletion of p53 or p21 we confirmed that p53 and p21 protect cells from apoptosis induced by cucurbitacins. These results demonstrated that sensitivity of human colon cancer cell lines to cucurbitacins depends on the kRas and p53/p21 status, and established that cucurbitacins can exert antitumorigenic activity in the absence of activated STAT3.

1. Introduction

Cucurbitacins are tetracyclic triterpenes isolated from plant families such as the Cucurbitaceae and Cruciferae that have been used for centuries as folk medicines [1]. Indeed, cucurbitacins have been shown to have anti-inflammatory, analgesic as well as cytotoxic activity *in vitro* and *in vivo* [2,3]. Cayaponia tayuya (Cucurbitaceae) is a climbing lignified plant with a large tuber that has long been used in the folk medicines of Brazil, Peru, and Colombia as an anti-inflammatory, antitumour and anti-rheumatic agent [4]. We recently isolated two cucurbitacins from the roots of Cayaponia tayuya,

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identified as 23,24-dihydrocucurbitacin B (DHCB) and cucurbitacin R (CCR) [3]. We demonstrated the anti-inflammatory, anti-allergic and anti-arthritic activity of DHCB and CCR *in vitro* and *in vivo*, due to their ability to inhibit the expression of TNFα in lymphocytes and in macrophages, and to interfere with the activity of the nuclear factor NF-AT [2,5,6].

The ability of cucurbitacins to inhibit the activity of COX2 [7] and the production of proinflammatory mediators such as iNOS and TNF [5] underlie their potent anti-inflammatory activity. Although these characteristics are likely to contribute to the ability of cucurbitacins to inhibit tumor progression, their antitumorigenic activity has been ascribed mainly to their propensity to inhibit JAK/STAT3 signaling, an important oncogenic pathway activated in a number of cancers [8–10]. Several cucurbitacins (such as cucurbitacin I, cucurbitacin Q and cucurbitacin B) were shown to inhibit phosphorylation of STAT3 and to induce apoptosis in v-Src transformed NIH3T3 cells, but had limited biological activity in cells with no activated STAT3 [11], which led to the conclusion that cucurbitacins exert antitumorigenic activity selectively in cells with activated STAT3.

Although activation of STAT3 has been demonstrated in primary colon tumors, the majority of established colon cancer cell lines lack constitutively activated STAT3 [12], suggesting that factors from the tumor microenvironment maintain constitutive activation of STAT3 in colon cancer. STAT3 transcriptional activity has been shown to be negatively regulated by PI3K and ERK signaling in melanoma cells [13]. Whether the STAT3 and Ras signaling pathways functionally interact in colon cancer cells, as we have shown for the STAT1 and Ras pathways [14], remains to be determined.

kRas mutations are found in 30–50% of primary colorectal cancers as well as in established colon cancer cell lines [15]. Ras genes encode small GTP-binding proteins that affect gene expression by acting as major switches in signal transduction processes, coupling extracellular signals with transcription factors [16–18]. Oncogenic forms of Ras are locked in their active state and transduce signals essential for transformation, angiogenesis, invasion and metastasis via downstream pathways involving the RAF/MEK/ERK cascade of cytoplasmic kinases, the small GTP-binding proteins RAC and RHO, phosphatidylinositol 3-kinase and others [19]. In addition to its role in tumor initiation, tumor maintenance and tumor progression, oncogenic Ras has been shown to modulate the responsiveness of cancer cells to a variety of chemotherapeutic and chemopreventive agents. We have shown that colon cancer cells that harbor mutant Ras are sensitized to apoptosis induced by butyrate [20], HDAC inhibitors [21] and 5-fluorouracil (5-FU) [22]. However, several clinical studies that have been conducted to examine the prognostic significance of Ras mutations in colorectal cancer have yielded inconclusive results [23,24].

The aim of the present study was to test whether DHCB and CCR, two cucurbitacins that we recently isolated from Cayaponia tayuya, inhibit proliferation and/or induce apoptosis in colon cancer cell lines, despite the fact that these cells do not express activated STAT3. Finally, we determined whether the presence of oncogenic kRas, a frequent genetic alteration in colon cancer, alters the responsiveness of cells to cucurbitacins.

2. Materials and methods

2.1. Cell lines and antibodies

The HCT116 human colon carcinoma cell line harbors an activating mutation in codon 13 of the kRas proto-oncogene and is wild type for p53. Isogenic cell clones with a disrupted mutant kRas allele [25], $p21^{-/-}$ HCT116 cells [26] and $p53^{-/-}$ HCT116 cells [27] were generated from the parental HCT116 cells by homologous recombination.

Cells were maintained in MEM medium supplemented with 10% FCS and antibiotics and their viability was assessed by the MTT assay (Boeringher, Mannheim, Germany). Rat intestinal epithelial cells (IEC-6), transfected with inducible kRas [28] were maintained in DMEM containing 400 μg/ml G418 and 150 μg/ml of hygromycin B. Expression of oncogenic Ras in these cells was induced by 5 mM IPTG. Immunoblotting was performed using standard procedures [14]. Antibodies specific for gelsolin were obtained from Sigma, and antibodies recognizing PARP, cleaved PARP, caspase-9, cleaved caspase-9, and cleaved caspase-3, were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies recognizing p53, PUMA, and p21 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). DHCB and CCR were isolated from the roots of Cayaponia tayuya as we described before [3], and cucurbitacin I (CCI) was purchased from EMD Biosciences (Darmstadt, Germany).

2.2. Transfection and reporter assays

To examine the effect of DHCB on the transcriptional activity of p21, we transiently transfected cells with 1 μg of plasmid containing a 2.4 kb genomic fragment of the p21 promoter cloned upstream of the LUC reporter gene [29], using the calcium phosphate transfection kit from Promega Biosciences (St. Louis, MO, USA). Twenty-four hours after transfection, cells were treated with DHCB at increasing concentrations for 12 h. Luciferase activity was normalized to TK-renilla activity to control for transfection efficiency.

2.3. Clonogenic and proliferation assay

To assess the effect of cucurbitacins on the colony forming ability of cancer cells, we performed clonogenic assay. Cells were seeded at a density of 200 cells per six well plate, and were treated with increasing concentrations of CCR or DHCB for 24 h. Cells were then washed and grown in complete media for seven days. Colonies were washed with PBS, fixed and stained with 6% glutaraldehyde and 0.5% crystal violet for 30 min at room temperature. Individual colonies with more than 50 cells were scored with Total Lab 1.1 software (Nonlinear Dynamics, Durham, NC, USA) and the surviving fraction was calculated.

Cell proliferation was assayed using a modified colorimetric Thiazoyl Blue Tetrazolium (MTT) assay. Briefly, 10^4 cells were plated in 96 well plates and treated with CCR (10–100 μM), DHCB (100 nM to 10 μM), and CCI (10 nM to 1 μM), and MTT activity was determined 24, 48, and 72 h after treatment.