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Original Articles

Aryl hydrocarbon receptor-dependent apoptotic cell death induced by the flavonoid chrysin in human colorectal cancer cells

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ABSTRACT

The polyphenolic flavone chrysin has been evaluated as a natural chemopreventive agent due to its anti-cancer effects in a variety of cancer cell lines. However, the mechanism of the chemopreventive effect has been not well established, especially in human colorectal cancer cells. We evaluated the chemopreventive effect of chrysin in three different human colorectal cancer cell lines. We found that chrysin treatment consequently reduced cell viability *via* induction of apoptosis. We identified that the involvement of up-regulation of pro-apoptotic cytokines tumor necrosis factor (*Tnf*) α and β genes and consequent activation of the TNF-mediated transcriptional pathway in chrysin-induced apoptosis. Using our generated AHR siRNA expressing colorectal cancer cells, we demonstrated that the chrysin-induced up-regulation of *Tnfα* and *β* gene expression was dependent on the aryl hydrocarbon receptor (AHR), which is a ligand–receptor for chrysin. Subsequently, we found that the AHR siRNA expressing colorectal cancer cells were resistant to chrysin-induced apoptosis. Therefore, we concluded that AHR is required for the chrysin-induced apoptosis and the up-regulation of *Tnfα* and *β* gene expression in human colorectal cancer cells.

Introduction

Despite advances in screening for and treatment of colorectal cancer, the impact of this malignancy on men and women both globally and in the United States remains substantial [1]. In 2014, there were an estimated 137,000 new cases of colorectal cancer in the United States with approximately 50,000 deaths, which ranks as the third leading cause of cancer death in both men and women [2]. The improved screening techniques for colorectal cancer have resulted in reduced incidence and mortality, with the advantage of detecting tumors at an earlier stage [1]. Additionally, advances in surgical intervention (i.e. total mesorectal excision in rectal cancer) with radiation therapy have reduced local recurrence rates and newer chemotherapeutic regimens have improved overall survival in patients with later stage disease [3–6]. Currently, strategies aimed at inhibiting malignant transformation have gained interest, because carcinogenesis within the colon and rectum is a multistep process that generally assumes a pre-cancerous state requiring sequential genetic defects to progress to invasive disease [7]. One putative avenue for disease prevention is characterized by the use of natural or synthetic agents to either suppress initiation of tumorigenesis or inhibit progression of premalignant cells to fully invasive complements, also known as chemoprevention [8].

Chemopreventive agents modulate tumorigenic pathways as opposed to chemotherapeutics that function to eliminate vast populations of premalignant or malignant cells [9,10]. Several studies have revealed an association between diet (dietary compounds) and cancer prevention [11–13]. The natural polyphenolic substances known as "flavonoids," which are prevalent in vegetables, fruits and nuts and constitute supplements with excellent safety profile and low toxicity, have gained significant interest by many in the field of chemoprevention [14,15]. Flavonoids encompass more than 4000 biologically active compounds with diverse functions such as enzyme inhibition, ligand-activation of signaling pathways and signal transduction [14,15]. Multiple subclasses of flavonoids possess anti-inflammatory, anti-oxidant and anti-proliferative capabilities [14,15]. Anti-carcinogenic properties have been attributed to free radical scavenging, modification of enzymes involved in carcinogen metabolism, inhibition of transcription factors and induction of apoptosis [14,15]. Epidemiologic investigations have highlighted the association

Abbreviations: AHR, aryl hydrocarbon receptor; AP-1, activator protein 1; ARNT, AHR nuclear translocator; BAD, Bcl-2-associated death promoter; CYP, cytochrome P450; DRE, dioxin-responsive element; ERK, extracellular signal-regulated kinase; FICZ, 6-formylindolo (3,2-b) carbazole; IEGs, immediate early genes; JNK, c-jun N-terminal kinase; LTα, lymphotoxin α; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κ-light-chain enhancer of activated B cells; PYCARD, PYD and CARD domain containing; rTdT, recombinant terminal deoxynucleotidyl transferase; siRNA, small interfering RNA; SRE, serum response element; SRF, serum response factor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCFs, ternary and complex factors; TNF, tumor necrosis factor; TNFR, TNF receptor; TUNEL, terminal deoxynucleotidyl transferase-dUTP nick end labeling.

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between reduced risk of development of various malignancies and flavonoid consumption [14,15].

One such flavonoid that has received considerable attention is chrysin, a compound present in honey, propolis and various plant extracts [16]. Among the molecular pathways investigated, chrysin has been shown to induce cell cycle arrest, increase degradation of hypoxia inducible factor α, inhibit tumor cell-induced angiogenesis, inhibit cell adhesion, and induce apoptosis in a variety of cancer cells [16]. However, despite numerous investigations for the anti-cancer effects of chrysin in several different cancer cells, the mechanism is not well established, especially in colorectal cancer cells. Therefore, we evaluated the anti-cancer effect of chrysin in human colorectal cancer cells. Additionally, we investigated the molecular mechanism of the chemopreventive effect by chrysin in colorectal cancer cells. We elucidated that chrysin induces apoptotic cell death in human colorectal cancer cells and that induction of pro-apoptotic cytokines tumor necrosis factor (TNF) α and β is involved in chrysin-induced apoptosis. Previous study demonstrated that chrysin is a natural agonist of the aryl hydrocarbon receptor (AHR) known as a xenobiotic receptor [17], therefore we hypothesized that activation of AHR would be required for chrysin's ability to kill colorectal cancer cells. As predicted, chrysin-induced apoptosis and induction of *Tnfα/β* gene expression are mediated *via* the AHR.

Materials and methods

Cell culture

Colon (HCT116, DLD1) and rectal (SW837) cancer cell lines were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 1% non-essential amino acids (Life Technologies), 1% penicillin–streptomycin (Life Technologies), and 1% glutamine (Life Technologies) at 37 °C and 5% CO_2.

Cell viability

Cells were seeded in 96-well plates with approximately 1.0×10^4 cells/well and incubated in DMEM supplemented medium for 24 hours. Cells were then treated with chrysin (Sigma-Aldrich, St. Louis, MO) (10 μM, 50 μM, 100 μM) or vehicle (DMSO) for 24 hours and the number of viable cells determined using an XTT proliferation assay (Roche Life Science, Indianapolis, IN). The absorbance (460 nm) and reference (750 nm) were measured using a spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA). For the fluorescence cell viability assay, cells were seeded in 96-well plates with approximately 1.0×10^4 cells/well and incubated in DMEM medium for 24 hours. Cells were treated with chrysin or vehicle for 6, 12, 24 and 48 hours. Cell viability was measured using CellTiter-Fluor™ cell viability assay kit (Promega, Madison WI). The fluorescence (excitation 390 nm, emission 460 nm) was detected using SpectraMax Plus 384 microplate reader (Molecular Devices).

Cytotoxicity and apoptosis assay

To investigate the mechanism of decreased cell viability induced by chrysin, we used the ApoTox-Glo™ Triplex Assay (Promega). Approximately 1.0×10^4 cells/well were seeded in a 96-well plate and treated with 100 μM chrysin or 0.1% DMSO for 6, 12, 24 and 48 h. Live-cells (cell viability) and dead-cells (cytotoxicity) were detected with treatment of fluorogenic peptide substrates glycylphenylalanyl-aminofluorocoumarin (GF-AFC) and bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AAF-R110), respectively. Fluorescence (GF-AFC (excitation 390 nm/emission 460 nm) and bis-AAF-R110 (excitation 485 nm/emission 520 nm)) was measured using SpectraMax Plus 384 microplate reader (Molecular Devices). Apoptosis activity was detected using Caspase-Glo® 3/7 Reagent (Promega). After addition of the reagent to cell culture medium, luminescence was measured by MicroLumat plus (Berthold).

TUNEL assay

DeadEnd™ Fluorometric TUNEL System (Promega) was utilized to evaluate cell apoptosis (DNA fragmentation) *via* incorporation of fluorescein-12-dUTP at 3′-OH DNA ends by recombinant terminal deoxynucleotidyl transferase (rTdT). Cells were treated with 100 μM chrysin or 0.1% DMSO for 48 hours and transferred to slides, which were then fixed, permeabilized, and treated with equilibration buffer followed by rTDT and nucleotide mix. The cells were then stained with propidium iodide (PI) and analyzed using fluorescence microscopy in which PI (apoptotic and non-apoptotic cells) and fluorescein-12-dUTP (apoptotic cells) were visualized. The number of terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) positive cells and total cell number were counted.

Gene expression analysis

Cells were treated with chrysin, 6-formylindolo (3,2-b) carbazole (FICZ) or vehicle (DMSO) as described. Total RNA was isolated from cells using the Qiagen RNeasy kit (Qiagen, Valencia CA). The isolated RNAs were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The mRNA levels were measured with TaqMan Universal PCR Master Mix (Applied Biosystems) and custom-designed probes (Assay ID: *CYP1A1*; *Hs01054797_g1*, *Tnf-α*; *Hs01113624_g1*, *Lt α(Tnf-β)*; *Hs04188773_g1*, *Ahr*: *Hs00169233_m1*, *c-fos*; *Mm00487425_m1*, *β-actin*; *Hs01060665_gl*). *β-actin* mRNA levels were measured as internal controls.

PCR array

Gene expression associated with apoptosis was evaluated using the RT^2 Profiler PCR array (PAHS-012Z, Qiagen). HCT116 cells were treated with 100 μM chrysin or 0.1% DMSO for 24 hours. Total RNA for RT^2 Profiler PCR array was extracted using RNeasy mini QIAcube kit. The data analysis was performed by web-based RT^2 Profiler™ PCR Array Data Analysis program. Genes that demonstrated a two-fold change or greater (chrysin (n = 4) vs. DMSO (n = 4), $p < 0.05$) were selected for further correlation analyses.

Stable si-RNA expression cell lines

For generation of small interfering RNA (siRNA) stable expression cell lines, HCT116 cells were transfected in 6-cm diameter dishes with 5 μg of pRNAT-U6-siAHR (5′-GGATCCCAAGATGGATCAATACTTCCACTTGATATCCGGTGGAAGTATTGATCCA TCTTTTTTTTCCAAAAGCTT-3′) or pRNAT-U6-siScramble (5′-GGATCCCACATGAT CGACTATAACACGT TTGATATCCGGTGGAAGTATTGATCCATCTTTTTTTTCCAAAAGCTT-3′) (Genscript) using Lipofectamine 2000 (Life Technologies). Six hours after transfection, cells were transferred to 15-cm diameter dishes and allowed to recover for a further 24 hours. The transfected cells were selected by culturing cells in a DMEM medium (with 10% fetal bovine serum) containing 900 μg/ml G418. After 5–7 days of selection, resistant colonies were isolated and expanded in DMEM medium containing 250 μg/ml G418. The resistant clones were screened by *Ahr* and *CYP1A1* mRNA levels using RT-PCR.

Luciferase reporter gene assay

To determine the alteration of TNF-mediated transcriptional regulation from treatment with chrysin, cells were transiently transfected with TNF signaling pathway analysis luciferase reporter vector (pNF-κB-RE LUC (pGL4.32), pSRE-LUC (pGL4.33) or pAP1-RE LUC (pGL4.44) (Promega)) and pTK-*renilla* luciferase plasmid (Promega) using Lipofectamine 2000 (Life Technologies). Six hours after transfection, cells were cultured with DMEM media containing 100 μM chrysin or 0.1% DMSO for 6, 12 and 24 hours. The luciferase activity was measured using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions and MicroLumat Plus luminometer (Berthold Technologies, Hartfordshire, UK).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 5, La Jolla, CA). Statistically significant differences between treatment and control groups were determined using unpaired t-test with Welch's correction. Data were presented as mean ± standard error of mean and were considered statistically significant when p value was <0.05.

Results

Chrysin induces cell apoptosis in colorectal cancer cell lines

The anti-cancer effects of chrysin have been observed in numerous cancer cell lines [16]. However, its effects in colon and rectal cancer cell lines have not been well studied. To evaluate chrysin effects on colorectal cancer cells, we first examined changes in cell viability with treatment with chrysin using HCT116, DLD-1 and SW837 colorectal tumor cell lines (Fig. 1A). With treatment of 10–100 μM chrysin for 24 hours, each cell line demonstrated a dose-dependent decrease in cell viability (Fig. 1A). Cell viability in all three cell lines was significantly decreased at 50 μM and 100 μM chrysin. HCT116 cells demonstrated the highest susceptibility to chrysin-induced cell death among the three cell lines with cell viability (61.4%