Food and Chemical Toxicology 97 (2016) 127–134

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Genistein induces apoptosis by down-regulating thioredoxin-1 in human hepatocellular carcinoma SNU-449 cells

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ARTICLE INFO

Article history:
Received 13 January 2016
Received in revised form
29 August 2016
Accepted 1 September 2016
Available online 3 September 2016

Keywords:
Genistein
Hepatocellular carcinoma
Apoptosis
Reactive oxygen species
Thioredoxin-1

ABSTRACT

Genistein (GEN), a natural isoflavonoid phytoestrogen, has anti-cancer activity against various types of cancers. However, GEN has not been thoroughly investigated in human hepatocellular carcinoma cells. In this study, we evaluated the anti-cancer effects of GEN on SNU-449 cells. GEN inhibited the proliferation of SNU-449 cells in a concentration-dependent manner. We observed the typical characteristics of apoptosis, such as DNA fragmentation and caspase-3 activation. To identify proteins related to GEN-induced apoptosis, we performed two-dimensional electrophoresis and identified differentially expressed proteins. Proteomic analysis showed that the antioxidant protein thioredoxin-1 was associated with GEN-induced apoptosis. GEN treatment decreased thioredoxin-1 levels and increased intracellular accumulation of reactive oxygen species. In addition, GEN activated apoptosis signal-regulating kinase 1, c-Jun N-terminal kinases (JNK) and p38. We also observed that pretreatment with the JNK and p38 inhibitors (SP600125 and SB203580) decreased GEN-induced cell death. These results indicate that GEN has potential antitumor effects against SNU-449 cells through the down-regulation of thioredoxin-1.

1. Introduction

Liver cancer is the sixth most common cancer and the second leading cause of cancer death worldwide in males (Torre et al., 2015). Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and treatment options for HCC are limited. Radiation therapies are not recommended because of the severe damage they inflict on liver tissues. Chemotherapies are only regionally effective (Newell et al., 2008). Moreover, the indiscriminate use of drugs, enhanced drug toxicity, and unexpected side effects make chemotherapy difficult for the patient.

Flavonoids are a group of common phenolic plant pigments that are present in a wide variety of plants, fruits, and vegetables. These compounds consist of flavones (7,8-benzoflavone or a-naphthoflavone), isoflavones, flavonols, flavanones, and chalcones. Genistein (GEN) is a natural isoflavone and is found in fruits, nuts, soybeans, and soy-based products (Snyder and Gillies, 2003). GEN induces apoptosis and cell cycle arrest in various cancer cells (Hsu et al., 2010; Hussain et al., 2012; Hwang et al., 2013; Mahmoud et al., 2015; Salti et al., 2000). However, the anti-HCC mechanism of GEN has not been clearly established. No studies have been conducted to determine the molecular mechanisms of its possible anticancer effect on HCC SNU-449 cells. Furthermore, how GEN induces apoptosis through a signal cascade in SNU-449 cells is unknown.

Protein expression profiling has enhanced the understanding of various types of cancer. In studies examining the cellular mechanisms of anti-cancer agents, this technique provides extensive information, enabling the detection of specific cancer biomarkers (Srinivas et al., 2002) and the mechanisms of various cancer agents (Bichsel et al., 2001). In this study, we performed protein expression profiling of GEN-treated SNU-449 cells using 2-dimensional electrophoresis (2-DE) proteomic analysis. Differentially expressed spots were identified and analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Using this technique, we detected the thioredoxin-1 (Trx1) protein, which has previously been implicated in reactive oxygen species (ROS) metabolism and apoptosis (Hwang et al., 2015; Kim et al., 2015; Kinnula et al., 2004; Pan et al., 2014).

Thioredoxins are low-molecular weight (10–12 kD) redox proteins found in both prokaryotic and eukaryotic cells (Powis and Montfort, 2001). There are two known forms of human thioredoxin: Trx1, which has a predominantly cytoplasmic, but also nuclear, localization (Gasdaska et al., 1994; Wei et al., 2000), and

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http://dx.doi.org/10.1016/j.fct.2016.09.003
0278-6915/

thioredoxin-2, which is found in the mitochondria (Spyrou et al., 1997). Trx1 regulates the DNA-binding and trans-activating activity of redox-sensitive transcription factors such as activator protein-1, glucocorticoid receptor, p53, and nuclear factor-κB (Abate et al., 1990; Grippo et al., 1983; Qin et al., 1994; Ueno et al., 2000). Trx1 also binds to apoptosis signal-regulating kinase 1 (ASK1) and protein kinases C to regulate their activity in a redox-dependent manner (Saitoh et al., 1998; Watson et al., 1999). Increased Trx1 expression has been linked to increased tumor-cell growth and suppressed apoptosis (Baker et al., 1997; Freemerman and Powis, 2000).

The objective of this study was to examine the anti-proliferative activities of GEN in HCC cells. To accomplish this, we investigated the cellular mechanisms related to apoptosis induced by GEN in SNU-449 cells.

2. Materials and methods

2.1. Cell culture

HCC cells (Huh7, SK-HEP-1, SNU-449) and normal human hepatocytes (CCL-13) were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained under standard conditions for temperature (37 °C), humidity (95%), and CO_2 (5%). Huh7, SK-HEP-1, and CCL-13 were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). SNU-449 cells were cultured RPMI 1640 (GIBCO) containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. At 70% confluence, the cells were harvested using 0.25% (w/v) trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA), and were then reseeded for expansion. One day after seeding, the media were replaced with fresh media containing dimethyl sulfoxide (DMSO)-dissolved GEN (Sigma-Aldrich). Control cells were cultured in medium containing the same amount of DMSO rather than GEN.

2.2. Cell proliferation assay

All cells were plated in 96-well plates at a density of 8×10^3 cells per well and incubated for 24 h. The cells were rinsed with PBS and grown in a medium containing various concentrations of GEN (0, 200, 400, 600 μM). We referred to other studies-related GEN to fix concentration of GEN (Salti et al., 2000; Thasni et al., 2008). After 24 h of treatment, the medium was removed and replaced with another medium containing 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (1 mg/mL), and the cells were incubated for 2 h at 37 °C. To assess the proportion of viable cells, formazan was solubilized in 200 μL DMSO. Plates were then shaken at room temperature for 30 min, and the level of formazan was measured using a spectrophotometer at 575 nm.

2.3. Caspase-3 assay

The activity of caspase-3 was determined using the caspase colorimetric assay kit (Sigma-Aldrich). This assay measures the cleavage of a specific colorimetric caspase substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). p-nitroaniline (pNA) is released from the substrate upon cleavage by caspase. Free pNA produces a yellow color that is monitored by a spectrophotometer. Caspase-3 activity was measured in the cell lysates. The cell pellets were incubated at 4 °C for 20 min with lysis buffer containing 50 mM HEPES (1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt), pH 7.40, 5 mM CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), and 5 mM DTT (1,4 dithio-DL-threitol). The lysed cells were centrifuged at 16,000 × *g* for 15 min at 4 °C, and the supernatants were analyzed immediately. The reaction mixture (total volume, 100 μL) contained 30 μL of cell lysate and 10 μL of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide in assay buffer, and the assay was carried out in a 96-well plate. To account for non-specific hydrolysis of the substrate, a control reaction mixture was prepared containing 30 μL of cell lysate, 10 μL of substrate, and 10 μL of the specific caspase-3 inhibitor acetyl-DEVD-CHO in assay buffer. Both mixtures were incubated for 90 min at 37 °C and the absorbance was read at 405 nm using a μQuant microplate reader (Bio-Tek, Winooski, VT, USA).

2.4. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling assay

A terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was also performed to detect the fluorescence of apoptotic cells using TdT enzyme with the fluorometric TUNEL system (Promega, Madison, WI, USA). Briefly, the cells grown on chamber slides were fixed with 4% methanol-free formaldehyde (pH 7.40) for 1 h, permeabilized using lysis buffer (1% Triton X-100 in 1% sodium citrate) for 15 min, and then treated with 50 μL TdT enzyme buffer, which was attached to strand breaks. Finally, labeled strand breaks were visualized by the attachment of fluorescein isothiocyanate-5-dUTP. All slides were counterstained using 4′,6-diamidino-2-phenylindole and detected using a fluorescence microscope (Nikon Eclipse TE 2000-U, Tokyo, Japan).

2.5. Annexin V/PI staining

We used a FITC Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) to confirm GEN-induced apoptosis. GEN-treated SNU-449 cells (1×10^6 cells) were washed with cold PBS and resuspended in 1 × binding buffer, followed by labeling with FITC Annexin V and incubation for 15 min at room temperature in the dark. After labeling, the cells were analyzed by flow cytometry (Beckman Coulter Brea, CA, USA).

2.6. Two-dimensional electrophoresis (2-DE)

SNU-449 cells (5×10^6 cells) treated at 400 μM concentrations of GEN for 24 h were harvested and lysed in a lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 0.5% pharmalyte) containing protease inhibitor (Sigma–Aldrich). Protein samples were loaded on the Immobiline DryStrip (18 cm, pH 3–10, GE healthcare, Little Chalfont, UK). IEF was performed in a Multiphor II IEF system (GE healthcare) at 24 °C for the first dimensional separation. After IEF, IPG strips were placed in equilibration buffer (75 mM Tris–HCl, pH 8.8, containing 6 M urea, 29.3% glycerol, 2% SDS and 0.02% bromophenol blue) for 10 min twice. The proteins were applied to 12.5% SDS–polyacrylamide gels for electrophoresis using Ettan DALTsix vertical electrophoresis system (GE healthcare). The SDS–polyacrylamide gels were visualized by coomassie staining to compare spot patterns between control and GEN-treated cells. Protein spots were extracted from 2-DE gels and identified by MALDI-TOF/MS, as described in a previous study (Yoo et al., 2009).

2.7. Determination of ROS level

Intracellular ROS generation was assessed using the stable nonpolar dye 5- (and-6)-carboxy-20,70-dichlorofluorescein