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Genistein synergizes with RNA interference inhibiting survivin for inducing DU-145 of prostate cancer cells to apoptosis

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ABSTRACT

To further investigate the effect of a combination of genistein with survivin of RNA interference on the proliferation and apoptosis of DU-145 cells, the effect of genistein on the proliferation of DU-145 cells was detected by the MTT method and cytometry, and the apoptosis of cells was observed with fluorescence microscopy. In order to test combined genistein with transfection of small interfering RNA (siRNA) against survivin, a survivin siRNA plasmid was constructed and transfected into DU-145 cells. Genistein inhibited proliferation and induced apoptosis of cancerous DU-145 and Hela cells, whereas genistein had minimal effects for normal L-O2 cells. The stable transfected cell lines of DU-145, knockdown survivin by siRNA, displayed stronger apoptotic than untransfected DU-145, the transfected cell of DU-145 treated with genistein demonstrated the inhibition of proliferation and induction of apoptosis significantly; it showed genistein synergistic effect with RNAi in survivin for inhibition of prostate cancer cells.

1. Introduction

The most intensively studied phytoestrogen group is that of the isoflavones, of which the effects of genistein (4,5,7-trihydroxyisoflavone) are the best-documented [1]. The present studies determined the effects of genistein on human prostate cancer cell lines *in vitro*. Genistein arrests cell cycle progression at the G2/M or G1/S phases. Genistein contributes to apoptosis and inhibits proliferation via promotion of antioxidant enzyme activities in prostate cancer cells of LNCaP and PC-3 [2], as well as DU-145 [3]. Genistein may also modulate correlative gene expression to promote cancer cell apoptosis through the influence of growth arrest and DNA damage-inducible gene 45 (Gad45) in DU-145 [4]. The mechanism of action of genistein is complex and includes several cellular pathways. In addition to its estrogenic and/or anti-estrogenic activities, genistein has been reported to inhibit steroidogenesis and block several protein tyrosine kinases, including epidermal growth factor receptor and src tyrosine kinases [5].

Survivin is an anti-apoptotic protein within the inhibitor of apoptosis proteins (IAPs) family and one of the most prominent cancer-associated genes that have been analyzed [6]. Survivin has been reported to inhibit cell proliferation and changes in cell cycle distribution of stable transfected cells of human breast cancer cell lines (MCF7, HEK293, HT29, and Hela) with specific small interfering RNA (siRNA) of survivin gene RNA interference (RNAi) [7]. The cells were blocked in the G0/G1 phase and sharply reduced in the G2/M phase so as to increase apoptosis of cells [8]. Survivin inhibits activation of caspases, and its overexpression can lead to resistance to apoptotic stimuli [9]. Caspases are a family of cysteine proteases and play

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; shRNA, small hairpin RNA; EGF, epidermal growth factor; PTK, protein-tyrosine kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IAP, inhibitor of apoptosis protein; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-bis(aminoethyl ether)-N,N-tetraacetic acid; MAPK, mitogen-activated protein kinases.

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essential roles in apoptosis. There are two types of apoptotic caspases: initiator caspases (caspases-2, -8, -9, and -10) and effector caspases (caspases-3, -6, and -7). The proteolytic activity of caspase-3 is a critical determinant of whether a cell commits to death, and as a result, multicellular organisms have evolved strategies to tightly regulate caspase-3 activity within the cell [10].

In this study, we tested the influence genistein extracted from soybeans on cell proliferation and/or apoptosis in human cancer cell lines (DU-145 and Hela) and a human hepatic normal cell line (L-O2). Furthermore, we carried out experiments on the role of genistein with survivin RNAi inhibiting survivin for inducing DU-145 of prostate cancer cell apoptosis to verify the protective effect of survivin expression. We adopted a plasmid that carried short hairpin RNA reversed to the survivin gene by RNAi to cause cell cycle arrest or apoptosis in DU-145, and combined genistein and survivin RNAi to observe the synergistic effect. The purpose of this study was to explain survivin expression in prostate cancer and to provide new direction for chemopreventive and/or chemotherapy against prostate cancer.

2. Materials and methods

2.1. Materials

2.1.1. Chemical materials

Genistein extracted from soybean (Gen, 4,5,7-trihydroxyisoflavone, 96% of purity, Shijiazhuang Biological Products Co., Ltd., Shijiazhuang, China) was dissolved in dimethylsulfoxide (DMSO; Sigma Co., Ltd. Steinheim, Germany) and diluted to final concentrations in each culture medium used (*vide infra*).

2.1.2. Plasmid and shRNA

Plasmid of U6 was constructed a recombinant encoding two survivin short hairpin RNA small hairpin RNA (shRNA) and named pU6 + 27-survivin, it was DNA template oligonucleotides corresponding to survivin gene which designed, fragment of 28nt, and synthesized as follows (Sangon Inc. Shanghai, China):

*Sal*I sense: loop antisense
5′-TCGACGAAGGACCACCGCATCTCTACATTCAAGAATT-
CAAGAGATTCTTGA ATGTAGAGATGCGGT
end *Xba*I
GGTCCTTTTTTTTT-3′
3′-GCTTCCTGGTGGCGTAGAGATGTAAGTTCTTAAGTTCT-
CTAAGAACTTACATCTCTACGCCACCA GGAAAAAA AAA-
GATC-5′

The above were subcloned into the SalI, XbaI and HindIII sites of pU6 + 27 plasmid (Promaga, USA), in order to be helpful in confirming the inserted sequence, we added a SalI site to the terminal of siRNA. Two pairs of survivin-specific siRNAs were selected in this study and an unrelated siRNA was used as negative control [11]. The oligonucleotide templates were then incubated in annealing buffer, first at 94 °C for 5 min and at 37 °C for night's lodging. pU6 + 27-survivin was already accomplished (Xiao Weihua's Laboratory of University of Science and Technology of China, USTC) and stored at −80 °C until they were used for transfections.

2.2. Cell culture

DU145 cells (androgen-independent human prostate cancer cell line, University of Science and Technology of China, USTC), Hela (human cervical cancer cell line, USTC) and L-O2 (normal human hepatic cell, Nanjing KeyGen Biotech. Co., Ltd., China) were, respectively, maintained in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Material Co., Ltd., China), each 100 U/ml of Penicillin/streptomycin (Invitrogen, USA), at 37 °C in the humidified atmosphere of a 5% CO_2 incubator. After about 80% of confluence, the cultured cells were digested using 0.25% trypsin (Amresco, USA) and subcultured.

2.3. Cell cycle analysis cell proliferation and viability assay

The effect of genistein on cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco USA) assay, based on the ability of live cells to cleave the tetrazolium ring in active mitochondria to a product molecule that absorbs at 570 nm of wavelength. Cells were plated in 96-well microtiter plates at an initial density of 1×10^4 cells per well.

DU-145, Hela and L-O2 of Cells were treated with genistein (15, 30, 60, 120 μM in medium) each, respectively, the blank was uniform concentration of DMSO. All treated cell were incubated for 48 h, then washed in PBS and cleared away supernatant fluid, 180 μL of fresh DMEM and 20 μL of MTT solution (MTT was dissolved in PBS at 5 mg/ml. Briefly) were added to each well, followed by 4 h of incubation. After incubation, MTT-containing medium was removed and 150 μL of DMSO was added to each well to dissolve formazan crystals. The concentration of formazan was quantified spectrophotometrically (BIO-TEK ELX800, Bio-Tek, Co., Ltd) at 570 nm. Cell proliferation was determined as above.

2.4. Cell calculation and cell modality observation

DU-145 and Hela were treated with 60 μM, 120 μM of genistein and incubated for 24 h, 48 h and 72 h, then cells were digested use 0.25% trypsin and living cell calculated by microscope; The same of DU-145 and Hela treated with 30 μM, 60 μM of genistein for 48 h, added 20 mg/mL of fluorescent dye (hoechst 33258, Sigma) and incubated at 37 °C for 20 min, observed by fluorescent microscope.

2.5. Western blotting analysis and activity assay toward caspase-3

DU-145 cells were harvested for 0, 1, 3, 6, 12, or 24 h times after genistein treatment (60 μM) and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, and 10 mM Tris–HCl [pH 7.4]) containing protease inhibitors. Cell debris was removed by centrifugation at 10,000*g* for 10 min at 4 °C. The resulting supernatants were