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ABSTRACT

Apoptosis, or programmed cell death, plays in important role in proliferative disorders, particularly cancer. A decrease in apoptosis allows cancer cells to grow out of control. These conditions make the apoptosis pathway an attractive target for new strategies in cancer therapy. The purpose of this research project was to investigate the anticancer potential of two compounds that occur naturally in common plants. All our experiments utilize fluorescent molecular probes measured in a fluorescent microplate reader or with a fluorescent microscope equipped for high-resolution, real-time, digital imaging. Using human MCF-7 breast cancer cells, two compounds were tested for their anticancer potential by measuring growth inhibition and apoptosis. Cucurbitacin E, a triterpene sterol found in various plants, has exhibited concentration-dependent growth inhibition due to cytotoxic cell death and apoptosis. The second compound, genistein, is one of the primary isoflavones found in soy. A diet high in soy products, like those of many Asian countries, has been shown to reduce the risk of breast cancer. We demonstrated that treatment with genistein resulted in concentration dependent growth inhibition with an effective concentration of 85μM. Cells treated with genistein also exhibited significant amounts of apoptosis, as determined by DNA fragmentation and morphological characteristics. Total methanol extracts of soy, obtained from different geographical locations, were assayed in the same manner. We found that these extracts were also capable of growth inhibition and that the extent of these effects varied between the extracts. The results presented support our conclusion that cucurbitacin E is highly cytotoxic and may not be suitable for targeting the apoptosis pathway, while genistein induces significant amounts of apoptosis by an unknown mechanism.

INTRODUCTION

Cells undergo the process of apoptosis, or programmed cell death, for various reasons. For example, apoptosis naturally occurs when cells are infected by viruses, or suffer irreparable damage. When cells of the body are enabled to bypass the apoptosis pathway, serious consequences result manifested in proliferative disorders, particularly cancer. A decrease in apoptosis allows cancer cells to grow out of control, resulting in

tumor formation. If the apoptosis pathway could be restored, transformed cells could be eliminated without inflammatory response or nonspecific drug interactions damaging adjacent normal tissue. These conditions make the apoptosis pathway an attractive target for new strategies in cancer therapy.

Development of cancer has not only been linked to genetic factors, but also environmental factors and personal habits, like exercise and diet. In Asian countries there are significantly fewer deaths from breast cancer in comparison to the United States and Western Europe. Various studies done over the past 20 years have shown a clear correlation between different lifestyles and the incidence of breast cancer, rather than genetics in these populations. In particular, individuals in these Asian countries consume 20-50 times more soy products in comparison to Americans (1, 2). Genistein (4',5,7-trihydroxyisoflavone), one of the primary isoflavonoids found in soy, has been shown to inhibit protein tyrosine kinase (3, 4) and inhibit growth of various carcinoma cell lines (1, 4, 5).

Cucurbitacins are triterpenes found in many plants that are believed to have medicinal properties. In particular, cucurbitacin E is also been shown to be cytotoxic in prostate carcinoma cells and is proposed to work by disrupting the F-actin cytoskeleton (6).

The question in our study was whether either of these compounds used apoptosis as a mechanism of cell growth inhibition. To answer this question, we treated MCF-7 breast carcinoma cells with the two compounds and determined the effective concentration resulting in growth inhibition of 50% of the control (EC_{50}). We determined the presence of apoptosis by measuring DNA fragmentation and morphological criteria. Our findings demonstrate that cucurbitacin E is highly cytotoxic, but does not induce apoptosis above base line level. Therefore, we concluded that it may not be suitable for further study as an anticancer agent. Genistein exhibited growth inhibition, along with significant amounts of apoptosis making it a suitable candidate for further testing. The soy extracts also exhibited growth inhibition, but the extent of inhibition was dependant on the origin of the extract.

MATERIALS AND METHODS

Chemicals

Cucurbitacin E and soy total methanol extracts were obtained from F. Halaweish (South Dakota State University). Genistein was purchased from Sigma Chemical (St. Louis, MO). Cell culture media and supplements were purchased from GIBCO. APO-BrdU TUNEL Assay Kit and Hoescht 33342 were obtained from Molecular Probes.

Cell Culture

MCF-7 breast carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). The cells are cultured in RPMI-1640 media supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100μg/ml streptomycin, and at 37° C in a humidified incubator with 5% CO_2. Growth inhibition assays were performed on 96-well plates (Falcon). Cells were trypsinized and plated at 1.5×10^4 (cucurbitacin E)

and 1 x 10^4 (genistein) cells/well 24 hours before treatment. Cells grown on four well chamber slides (Falcon) were used for digital imaging.

GROWTH INHIBITION ASSAYS

Cucurbitacin E and Genistein

Fresh media containing cucurbitacin E at concentrations of 28, 56, 113, 225, 450 nM and a control containing the vehicle (0.1% dimethylsufoxide) were added to the 96-well plates and incubated for 1, 2, and 3 days.

Similarly, fresh media containing genistein at concentrations of 10, 25, 50, 100, 200, 400μM and controls containing the vehicle (0.1, 0.2, or 0.4% DMSO for 10-100μM, 200μM and 400μM, respectively) were added to the 96-well plate and incubated for 1, 3, and 6 days.

After treatment cells were stained with Heochst 33342 (10μg/ml) in phosphate buffered saline (PBS) for 30 minutes, washed with PBS, and read on the FLx800 (Bio-Tek Instruments Inc.) fluorescent microplate reader and analyzed using KC Junior software.

Soy Extracts

Four total methanol extracts of soy containing unknown concentrations of genistein were dissolved in DMSO at a concentration of 25 mg/ml. Fresh media containing each extract at a final concentration of 250μg/ml along with a control containing the vehicle (1% DMSO), and a trial of 100μM (27μg/ml) genistein for comparison were added to a 96-well plate (plated as before) and incubated for 3 days. The treated plates were then analyzed in the same manner as above.

Measurement of Apoptosis

Twenty-four hours after plating, MCF-7 cells were treated in four-well chamber slides with a control containing the vehicle and either 113 nM cucurbitacin E (incubated 1, 2, and 3 days), or 100μM genistein (incubated 1, 3, and 6 days). After treatment, slides were fixed with 3.7% buffered formaldehyde, washed with PBS, stained using the APO-BrdU TUNEL Assay Kit according to manufacturer's instructions, and immediately coverslipped with Fluoromount G (Southern Biotechnology Associates Inc, Birmingham, AL). The slides were analyzed using a Nikon Eclipse TE-300 inverted microscope equipped for fluorescence digital ratiometric imaging (Fryer Co., Huntley, IL). The images were captured using a Nikon Super Fluor 40x oil immersion objective and CoolSnap FX digital CCD camera (Photometrics, Trenton, NJ), (495nm excitation, 520 nm emission) and stored for analysis. Imaging analysis was performed using MetaMorph 5.0 software (Universal Imaging Corp.). Apoptotic indices were calculated by counting number of apoptotic cells, and the total number of cells in 3-4 randomly chosen fields of view.

RESULTS

Cucurbitacin E

MCF-7 cells treated with cucurbitacin E exhibited dose dependant growth inhibition (Figure 1A), supporting other groups (2) findings of growth inhibition on various different carcinoma cell lines. We estimated an EC_{50} of 100 nM after 3 days of incubation (Figure 1B). Under a light microscope morphological changes were also evident in treated cells characteristic of necrotic cells (not shown).

TUNEL assay was performed to determine what percentages of cells were undergoing apoptosis. Staining revealed only base line levels of apoptosis comparable to that found in the control cells.

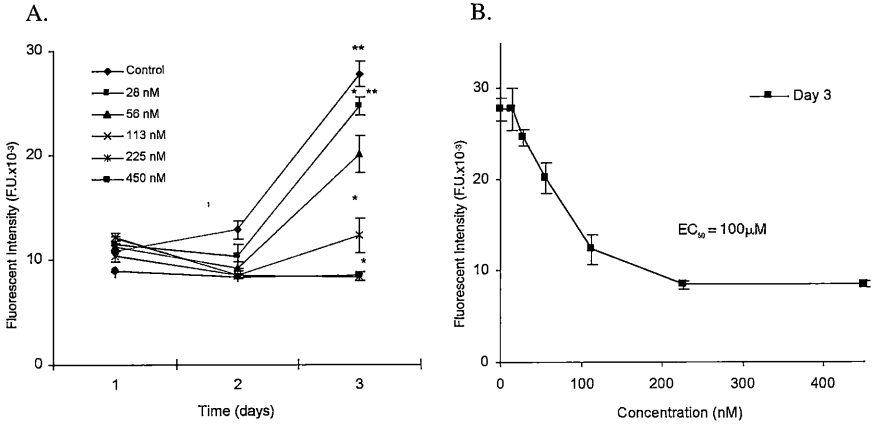

Figure 1. Growth Inhibition assay for MCF-7 cell treated with various concentrations of cucurbitacin E for 1, 2, and 3 days.

Values are means ±SEM. (*), differs from the corresponding control, P<.05. (**), day 3 mean is different from day 1 mean in same treatment group, P< .05. Growth was measured staining live cells with DNA stain Hoechst 33342.

Genistein

MCF-7 cells treated with genistein exhibited a dose dependant growth inhibition (Figure 2A). We found an EC50 of 85μM after 6 days of incubation (Figure 2B). We also observed growth stimulation at low concentrations (< 25μM) of genistein.

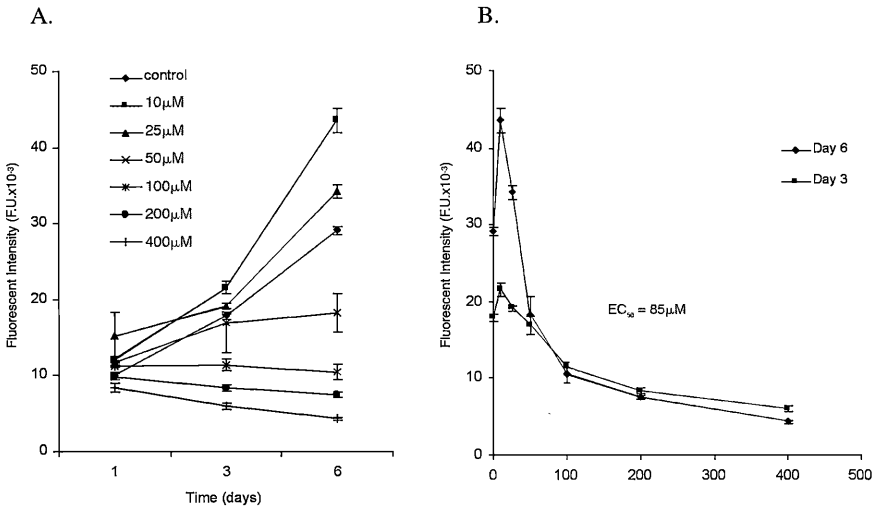

Figure 2. Growth Inhibition assay for MCF-7 cell treated with various concentrations of genistein for 1, 3, and 6 days.
Values are means ±SEM. All treatment group's means differ from control in day 6, P<.05. Growth was measured staining live cells with the DNA stain Hoechst 33342.

Results from the TUNEL assay showed significant apoptosis present in the treated wells after 3 and 6 days of incubation in comparison to the control (Table 1). Morphological appearance (rounded appearance, cell shrinkage, nuclear blebbing) of treated cells were characteristic of apoptosis, further supporting that apoptosis was present in treated cells (Figure 3A-D).

Treatment/ Time	Control		100 M	
	Day 3	Day 6	Day 3	Day 6
Apoptotic index, %	< 1	< 1	13.6 ± 3.1	29.3 ± 9.5

Table 1. Genistein treatment induces apoptosis in MCF-7 cells.
Apoptotic cells were counted in 3-5 randomly chosen fields of view for each day and treatment. Apoptotic indices were calculated by dividing the number of apoptotic cells by the total number of cells; means ±SEM.

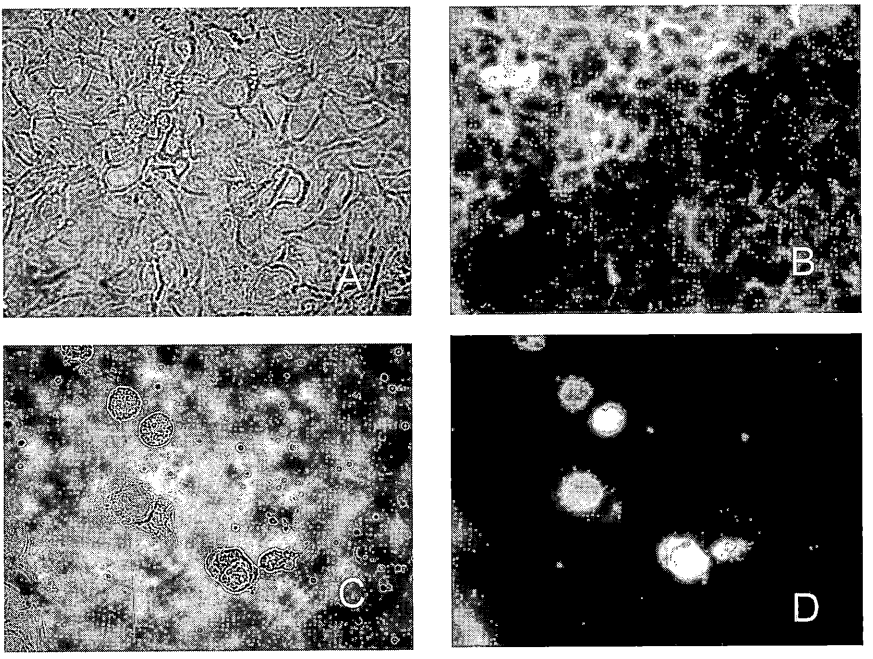

Figure 3A-D. Apoptosis in MCF-7 cells induced by genistein treatment.
Immunofluorecence detection of apoptosis with the APO-BrdU TUNEL Assay Kit. (A) light image of control cells after 6 days of growth, (B) corresponding fluorescence image of control cells. (C) Light image of cells treated with 100μM genistein for 6 days, (D) corresponding fluorescent image.

Soy Extracts

Treatment with soy extract IV (250μg/ml) resulted in growth inhibition similar to 100μM (27μg/ml) genistein. Extract I (250μg/ml) also showed some growth inhibition (Figure 4).

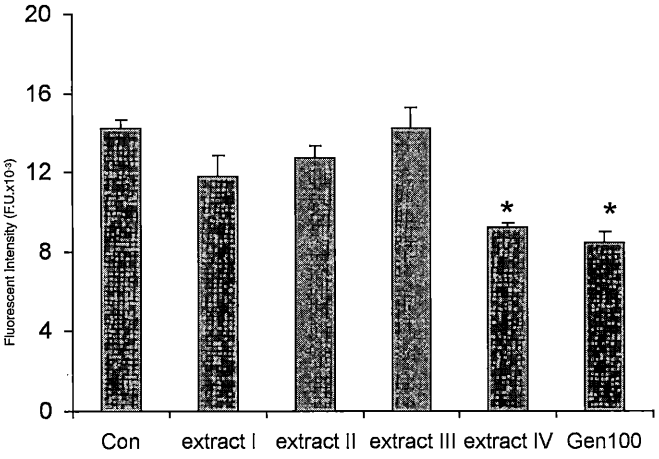

Figure 4. Inhibition assay of four soy extracts compared to 100μM treatment of genistein. Extracts I, II, III and IV derived from soy of maturity groups 3 (CF461), 4 (KS 4202), 4 (KS 5502N), and 5 (MGS). Values are means ±SEM. (*), mean differed from control groups mean by P<.05.

DISCUSSION

When screening compounds for therapeutic potential against cancer, desirable characteristics include growth inhibition at relatively low concentrations and specific targeting of transformed cells leaving remaining tissue unharmed. One drawback of many cancer therapies currently used is the inability of many effective drugs to do the latter efficiently, leaving the patient to endure the harsh side effects. One method of avoiding this would be to find a compound that can induce natural cell death through apoptosis in transformed cells. Although cucurbitacin E's mechanism of growth inhibition occurs under low (nanomolar) concentrations, our data indicates that it does not target the apoptosis pathway. Therefore the compound's ability to differentiate between normal and transformed cells is questionable, possible limiting its capability as an anticancer agent.

Genistein induces growth inhibition, although higher concentrations (micromolar) are needed to see the effect. This is supportive of results found by other groups (1, 4, 5) on various carcinoma cell lines. In addition, genistein does appear to target the apoptosis pathway (See Table 1) making it a much more appealing anticancer agent. The next step

in investigation of genistein will be to elucidate the mechanism of apoptosis and whether this mechanism will result in targeting of transformed cells. Other characteristics of genistein needed to be taken under consideration when considering it as an anticancer agent, include its estrogenic effects. Observed in this study and others (5, 7), low concentrations of genistein stimulates cell growth. Hsieh et al. found that genistein at low concentrations in fact could act as a weak estrogen, which may be associated with increased risk of breast cancer. It has also been shown that in vivo this leads to mammary gland differentiation in rats, which when administered at a young (prepuberty) age resulted in reduced incidence and multiplicity of breast tumors (2). Without a doubt, finding the mechanism by which genistein affects mammary tissue will be a key to understanding these diverse effects.

Treatment with the total methanol extracts of different soy maturity groups resulted in a spectrum of growth inhibition. While some extracts did not show any growth inhibition, extract IV showed significant inhibition comparable to that by 100 μM genistein (see Figure 4). Extract I showed mild effects of growth inhibition, but with more variability resulting in an average not statistically significant from the control. These results indicate that soy, grown under different environmental conditions may contain differing amounts of genistein and other bioactive compounds. It also represents yet another variable when studying how consumption of soy leads to reduced incidences of cancer.

In conclusion, our data clearly demonstrates that cucurbitacin E inhibits growth in a dose-dependant manner on mammary carcinoma cells. Its mechanism appears due to its high cytotoxicity and not the apoptosis pathway. Genistein plainly induces apoptosis in mammary carcinoma cells resulting in growth inhibition. The mechanism of apoptosis is still to be elucidated and may be a target for anticancer therapy. Extracts of soy, containing undetermined amounts of genistein, also show growth inhibition at different levels contingent on the geographical location from which the soy was obtained.

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