Abstract. Glycyrrhetinic acid is an active triterpenoid metabolite of glycyrrhizin abundantly present in licorice roots. Glycyrrhetinic acid exists as α and β stereo-isomeric forms. Both stereo-isomeric forms are known to have anti-inflammatory and anticancer activity. However, the effects and anticancer mechanism of α glycyrrhetinic acid in prostate cancer cells has not yet been evaluated. Therefore, we investigated the growth inhibition, induction of apoptosis and the anticancer mechanisms of 18α-glycyrrhetinic acid (AGA), on the androgen-independent metastatic prostate cancer cell line DU-145. Our results showed that AGA inhibited proliferation and growth of these cells by inducing apoptosis as determined by Annexin V and flow cytometry analyses. Our studies also showed that HUVEC tube formation was drastically reduced by down-regulation of inflammation-related genes of C30 are in the same plane while in AGA, they are not in same plane (20). BGA has been more extensively studied because of its abundance in root extract. Though it was widely believed when cultured in conditioned medium of AGA-treated DU-145 cells. In addition, AGA treatment prevented the invasion of DU-145 prostate cancer cells on matrigel coated transwells via down-regulation of NF-κB (p65), VEGF and MMP-9 expression. Furthermore, AGA treatment also down-regulated the expression of pro-inflammatory cytokine/growth factor genes HMGB1, IL-6 and IL-8 in DU-145 cells. Interestingly, AGA simultaneously upregulated the expression of non-steroidal anti-inflammatory gene-1 (NAG-1) in DU-145 cells suggesting its anti-inflammatory activity on prostate cancer cells. Taken together, the results of this study suggest that AGA may be a promising anticancer agent that merits further investigation for the chemoprevention and treatment of prostate cancer.

Introduction

Prostate cancer is the most frequently diagnosed non-cutaneous malignancy and the second leading cancer-related cause of death in men, responsible for nearly 30,000 deaths per year in the United States (1). It was estimated that prostate cancer would be responsible for 28% (186,320) of all newly diagnosed cancers by 2010. Primary stages of the disease can be treated with surgery, androgen ablation and or radiation therapy. Patients undergoing hormonal therapy eventually develop aggressive hormone unresponsive disease. Hence, the major focus in prostate cancer research is the discovery of better chemotherapeutic agents for the advanced hormone-resistant, metastatic form of this disease.

Licorice root extract, obtained from Glycyrrhiza glabra, is a well known oriental herbal medicine that has been most frequently prescribed for the treatment of various diseases. Chinese medicinal practitioners recommend licorice root for its life-enhancing properties and cure of injury or swelling and for detoxification (2,3). It is also used as a flavoring and sweetening agent in drug preparations, tobacco, chewing gums, candies and beverages (4-6). The major active component of licorice root is glycyrrhizin, an oleanane-type triterpene saponin which constitutes 2-24% of total dry weight (7). Glycyrrhizin is extensively reported to have several pharmacological activities including anti-inflammatory (8), immunomodulatory (9), anti-ulcer (10) and anti-tumorigenic activities (11,12). Glycyrrhizin also has antiviral activity against hepatitis B virus, HIV (13,14) and severe acute respiratory syndrome (SARS)-associated coronavirus (15).

When orally administered, before absorption, human intestinal bacteria completely convert glycyrrhizin to its aglycone metabolite glycyrrhetinic acid (16), which exhibits higher bioactivity than its precursor (17). Like glycyrrhizin, glycyrrhetinic acid can exist as two stereoisomers, the trans form 18α-glycyrrhetic acid (AGA) and the cis form 18β-glycyrrhetic acid (BGA) (18,19). Major structural differences between 18α- and 18β-glycyrrhetic acids lie in the spatial orientation of hydrogen atom of C18, in BGA hydrogen atom of C18 and the carboxyl of C30 are in the same plane while in AGA, they are not in same plane (20). BGA has been more extensively studied because of its abundance in root extract. Though it was widely believed...
that BGA has high bioactivity, some studies showed that AGA has comparable bioactivity to BGA (21,22). In vivo studies using mouse model demonstrated that AGA has more effective anti-inflammatory potential than BGA (22,23). AGA is found to be very effective in inhibiting skin tumor initiation and promotion (24). In this study, we have investigated the anticancer activity and mechanisms of AGA using DU-145 prostate cancer cells.

Materials and methods

Chemicals and reagents. Fetal calf serum (FCS), RPMI-1640 and minimum essential medium (MEM) were obtained from American Type Cell Culture (ATCC, Manassas, VA). 18α-glycyrrhetinic acid (AGA) was purchased from Sigma-Aldrich (St. Louis, MO). Cell viability assay kit was purchased from Dojindo Molecular Technologies Inc., (Gaithersberg, MD). Annexin V-FITC apoptosis detection Kit was obtained from MBL International (Woburn, MA).

Cell lines and cell culture. LNCaP, DU-145 and HUVEC cell lines were obtained from ATCC. LNCaP and DU-145 cells were cultured in RPMI medium supplemented with 10% FCS and 50 µg/ml gentamycin. HUVEC were cultured in EBM-2 medium (Lonza Walkersville Inc., MD) supplemented with 10% fetal bovine serum (FBS), hydrocortisone, basic human fibroblast growth factor (hFGF-b), vascular endothelial growth factor (VEGF), insulin-like growth factor (R3-IGF-1), ascorbic acid and human epidermal growth factor (hEGF). For tube formation assay, HUVEC were grown in conditioned medium of DU-145 cells. For all experiments, 1x10^5 cells/ml were seeded and grown for 24 h before experimental treatments. Cells were maintained at 37°C, 5% CO₂ environment.

Cell viability assay. LNCaP and DU-145 cells were treated for different periods of time with different doses of AGA. Cell viability was measured using a CCK-8 kit purchased from Dojindo Molecular Technologies Inc. Briefly, cells were cultured in 96-well plates in the presence of 10 µl aliquots of AGA at various concentrations ranging from 10 to 500 µM. Following exposure to AGA, 10 µl of CCK-8 solution was added to each well, and the plates were incubated for 3 h at 37°C and read immediately at 450 nm using a microplate reader (Bio-Rad, Richmond, CA).

Assessment of apoptosis in DU-145 cells by fluorescent microscopy and flow cytometry. DU-145 cells were stained with an Annexin V-FITC apoptosis detection kit (MBL) to determine whether cells are undergoing apoptosis. Propidium iodide staining was used as a control to differentiate cells undergoing necrosis. DU-145 cells were seeded in tissue culture and grown for 24 h before experimental treatments. Cells were maintained at 37°C, 5% CO₂ atmosphere.

Western blot analysis. DU-145 cells treated with 100 µM AGA for 24 h were lysed with sample solubilizing buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-p65 (MBL international Inc.), anti-MMP-9 (Cell Signaling Technologies), anti-VEGF (Fisher Scientific) or anti β-actin antibodies (Sigma-Aldrich). Untreated cells served as controls.

Real-time quantification of cytokine genes. Quantification of gene expression of HMGBl, IL-6, IL-8 and NAG-1 was determined using real-time PCR technique. For gene expression studies, DU-145 cells were incubated for 48 h in presence of 100 µM AGA. Following treatment, the cells were lysed with TRIzol reagent and the RNA extracted. Untreated cells served as controls. From the RNA samples, cDNA was synthesized using SA Biosciences PCR kit. A SYBR green based gene specific real-time PCR quantification kit (SA Biosciences) was used to analyze the expression of HMGBl, IL-6, IL-8 and non-steroidal anti-inflammatory gene-1 (NAG-1) genes. Real-time amplification of genes was performed in Applied Biosystems 7300 real-time PCR instrument.
Statistical analyses. Data were compared using Mann-Whitney rank sum test using SigmaStat program (Jandel Scientific, San Rafel, CA). P<0.05 was considered statistically significant.

Results

AGA inhibited growth and proliferation of DU-145 cells in a dose-dependent manner. LNCaP and DU-145 cells were treated with varying concentrations of AGA for 48 h. Cell viability assay revealed that AGA treatment induced a dose-dependent reduction in cell viability starting at 100 µM concentrations on both androgen-dependent LNCaP and androgen independent prostate DU-145 cancer cells (Fig. 1). Interestingly, DU-145 cells were more sensitive to AGA treatment and hence further characterization studies were focused on DU-145 cells.

AGA induces apoptosis in DU-145 cells. To further characterize the effects of AGA on DU-145, Annexin V-FITC and propidium iodide staining was used to determine the cause of cell death. DU-145 cells were treated with 100 or 200 µM AGA for 48 h and stained with Annexin V-FITC and propidium iodide to visualize the cells under fluorescent microscope. Fluorescent microscopic analysis demonstrated that DU-145 cells treated with AGA were undergoing apoptosis and positively correlated with dose of AGA exposure (Fig. 2).

Similarly, flow cytometry analyses also confirmed that AGA treatment induced apoptosis in DU-145 cells (Fig. 3)

AGA inhibits tube formation of HUVEC and invasion of DU-145 cells in vitro. DU-145 prostate cancer cells express and secrete angiogenic factors for angiogenic process involving endothelial cells. To determine the anti-angiogenic activity of AGA, the conditioned medium of DU-145 cells treated with 100 µM AGA was used to culture HUVEC on matrigel for 24 h. These results showed that HUVEC grown in conditioned medium of AGA treated DU-145 cells were severely defective in tube formation on matrigel (Fig. 4A). However, the tube formation was not affected in the HUVEC when cultured in the control conditioned medium of DU-145 cells. We also tested the anti-invasive potential of AGA on DU-145 cells using matrigel coated transwell filters. These results also inferred that AGA treatment significantly prevented the number of DU-145 cells invading the transwell filters coated with matrigel compared to untreated cells (Fig. 4B).

AGA treatment down-regulates the expression of p65, VEGF and MMP-9 in DU-145 cells. NF-xB (p65) plays critical role in angiogenesis and invasion of prostate cancer cells by regulating the expression of key molecules, VEGF and matrix metalloproteinase-9 (MMP-9). Immunoblot analysis of DU-145 cells

Figure 2. Annexin V-FITC detection of apoptosis in prostate cancer cells: DU-145 cells (10^4 cells/ml) cultured in tissue culture slides were treated with AGA for 48 h. Untreated cells incubated in DMSO alone served as control. Following treatment, cells were stained with Annexin V-FITC and propidium iodide. The cells were visualized under a fluorescence microscope. Results show that prostate cancer cells treated with AGA undergo apoptosis in a dose-dependent manner compared to control cells.

Figure 3. AGA induces cell death in DU-145 prostate cancer cells. DU-145 cells treated with 100 µM AGA for 48 h were stained with PI and subjected to flow cytometry analysis. Results showed that 34% of DU-145 cells were dead (M1) while only 3% of cells were dead in the control DU-145 group. Results are representative of one of three similar experiments.
treated with AGA showed a marked reduction in the expression of p65, VEGF and MMP-9 (Fig. 4C) underscoring its anti-angiogenic and anti-invasive properties on DU-145 prostate cancer cells.

**Effect of AGA on the expression of inflammatory genes.** Several inflammatory-related genes have been linked with initiation and progression of prostate and other types of cancers (29,30). Previous studies showed that inflammatory cytokines IL-6 and IL-8 are associated with augmented growth (31) and elevated metastatic potential (31-33). Similarly, over-expression of nuclear binding protein and proinflammatory mediator HMGB1 has been implicated in proliferation and metastasis of many cancer types, including prostate cancer (34). Hence, we studied the effect of AGA on inflammatory genes in DU-145 cells by quantitative real-time RT-PCR. Our results showed that AGA exerts a negative effect on expression of HMGB1, IL-6, and IL-8 while activating NAG-1 expression in DU-145 cells.

**Discussion**

The number of men affected with prostate cancer is growing rapidly as the population of males over the age of 50 raises worldwide. Chronic inflammation caused by both hereditary and environmental factors has gained attention as a potential important element in the progression of cancer (35). Hence, in the present study we have tested the effects of a natural product AGA, an anti-inflammatory agent, on the androgen independent metastatic DU-145 prostate cancer cells.

Glycyrrhetinic acid was proven to inhibit the Epstein-Barr virus associated early antigen induction, ornithine decarboxylase and phospholipid metabolism controlled by tumor promoters (36-39). Further studies showed that glycyrrhetinic acid could also inhibit the tumor promoting activity of 12-O-tetradecanoylphorbol-13-acetate and teleocidin in mouse skin (36). Glycyrrhetinic acid has negative effect on growth and melanogenesis in cultured B16 melanoma cells and, 20 times lesser concentration of glycyrrhetinic acid than that of glycyrrhizin was needed to cause the same effect (40). Recently, we reported that glycyrrhizin could induce growth inhibition on prostate cancer cells (11). In the current study, our results show that AGA has potent anti-proliferative effects on both LNCaP and DU-145 prostate cancer cells. Interestingly, our
results showed that androgen-independent DU-145 cells were more sensitive to AGA treatment compared to androgen dependent LNCaP cells suggesting AGA might have differential effects on the stage and type of prostate cancer cells.

Following androgen ablation treatment, androgen-dependent prostate cancer cells cease to proliferate and undergo apoptosis. However, due to the heterogeneous presence of androgen-independent cells evolved from androgen-dependent cells, these cells continue to proliferate and their proliferation rate exceeds the apoptosis rate (41). Hence, an effective chemotherapeutic agent should not only limit proliferation, but should be capable of activating programmed death of androgen-independent metastatic prostatic cancer cells. As exposure of AGA to DU-145 reduced the proliferation and growth of these androgen-independent metastatic prostate cancer cells, we have analyzed whether apoptosis is involved in the reduction of cell viability using fluorescent microscopy. Binding of Annexin V to AGA treated DU-145 cells confirmed the pro-apoptotic potential of AGA. Flow cytometric analysis of DU-145 cells treated with AGA also confirmed that AGA induces apoptosis. Previous studies showed that AGA can induce apoptosis in MCF-7 breast cancer cells (21). Similarly, glycyrrhetinic acid induced apoptosis in human hepatoma (HLE), promyelotic leukemia (HL-60) and stomach cancer (KATO III) cells in a dose- and time-dependent manner (42). Thus, our study reiterates the apoptotic potential of AGA against the viability of cancer cells. Angiogenesis and invasion process plays a key role in the establishment and distant metastases of prostate cancer cells (43,44). Our preliminary results show that AGA has the ability to prevent both angiogenesis (tube formation of HUVEC) and invasion of DU-145 advanced prostate cancer cells mainly by targeting p65, VEGF and MMP-9 expression. Over-expression of p65, VEGF and MMP-9 is critical for the progression of prostate cancer. Therefore, AGA may be a promising anti-cancer agent which may be beneficial in the prevention and treatment of prostate cancer.

Recent studies showed that inflammation plays an important role in the initiation and progression of several kinds of cancers including prostate (45,46). Inflammatory cells and several mediators of inflammation including cytokines and chemokines promote the microenvironment required for cancer development. The pro-inflammatory cytokine levels, such as IL-6 and IL-8, are elevated in serum of men with advanced hormone sensitive prostate cancer (47-49). As inflammation and cell proliferation are associated with the development of prostate cancer, chemotherapeutic agents having anti-inflammatory as well as pro-apoptotic potential could be advantageous in cancer treatment. Previous studies showed that similar to that of glucocorticoid, AGA also has anti-inflammatory potential (22). Hence, we studied the effect of AGA over the production of proinflammatory mediators HMGB1, IL-6 and IL-8 in DU-145 cells.

Over-expression of HMGB1 is reported in prostate cancer cells (50,51) and depletion of HMGB1 expression induces apoptosis in prostate cancer cells (52). HMGB1 is a potent attractant for inflammatory cells including neutrophils, monocytes and macrophages as well as inducer of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6 and IL-8 production (53,54). Interestingly, HMGB1 is also an angiogenic switch molecule (55) and NF-kB interacting molecule (56) thus possibly regulating the expression of VEGF in DU-145 cells. IL-6 acts as an autocrine and paracrine growth factor for androgen-independent prostate cancer cell lines and depletion of IL-6 rendered these cells sensitive to chemotherapeutic agents (57). IL-8, another important pro-inflammatory cytokine, modulates the growth and metastasis of androgen-independent prostate cancer cells and confers chemotherapeutic resistance to them (58). Our results showed that AGA could decrease the expression of HMGB1, IL-6 and IL-8 in DU-145 cells. Interestingly, AGA activated the expression of NAG-1, an anti-inflammatory and apoptosis related gene in DU-145 prostate cancer cells. Thus, AGA could target multiple mechanisms that promote prostate carcinogenesis and progression.

In conclusion, AGA, a glycyrrhizin metabolite, is able to suppress the proliferation of androgen-independent metastatic prostate carcinoma cell line DU-145 through the induction of apoptosis in a dose-dependent manner. In addition, our studies also showed that AGA inhibited the tube formation of endothelial cells and prevented the invasion of DU-145 cells in vitro. Besides, AGA could down-regulate the expression of inflammatory mediators HMGB1, IL-6 and IL-8. Further investigations are necessary to confirm the potential of AGA as a therapeutic agent against prostate cancer.

Acknowledgements

This study was supported in part by funding received from Excellence in Academic Medicine (EAM) award of Swedish American Health System and grant no. 141287 from the American Cancer Society, Illinois Division, Inc. awarded to M.G.

References