Food and Chemical Toxicology 50 (2012) 4068–4076

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Mechanisms of natural brassinosteroid-induced apoptosis of prostate cancer cells

Jana Steigerová [a,b,*], Lucie Rárová [c], Jana Oklešťková [d], Kateřina Křížová [a], Monika Levková [a,b], Michaela Šváchová [e], Zdeněk Kolář [a,b], Miroslav Strnad [c,d]

[a] Laboratory of Molecular Pathology, Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic
[b] Institute of Molecular and Translation Medicine, Faculty of Medicine and Dentistry, Palacký University and Faculty Hospital in Olomouc, Puškinova 6, 775 20 Olomouc, Czech Republic
[c] Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Growth Regulators, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic
[d] Laboratory of Growth Regulators, Institute of Experimental Botany ASCR & Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic
[e] Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University and University Hospital Olomouc, I.P. Pavlova 6, 775 20 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Received 19 August 2011
Accepted 15 August 2012
Available online 23 August 2012

Keywords:
Apoptosis
Brassinosteroids
Cell cycle
Hormone-sensitive/insensitive prostate cancer cells

ABSTRACT

Brassinosteroids (BRs) are a group of polyhydroxylated sterol derivatives with important regulatory roles in various plant physiological processes. The aim of this study was to examine the mechanism of the anti-proliferative activity of natural BRs 28-homocastasterone (28-homoCS) and 24-epibrassinolide (24-epiBL) in hormone-sensitive and -insensitive (LNCaP and DU-145, respectively) human prostate cancer cell lines. The effects of BRs on prostate cancer cells were surveyed using flow cytometry, Western blotting, TUNEL, DNA ladder assays and immunofluorescence analyses. The studied BRs inhibited cell growth and induced G_1 blocks in LNCaP cells accompanied by reductions in cyclin D_1, CDK4/6 and pRb expression. Following BR treatment of DU-145 cells, increases in proportions of cells in the G_2/M phase of cell cycle were observed, accompanied by down-regulation of cyclins A and B_1. Changes in AR localization patterns in LNCaP cells treated with BRs were shown by immunofluorescence analysis. Furthermore, apoptotic detection methods demonstrated induction of apoptosis mediated by BRs in both cell lines, although changes in the expression of apoptosis-related proteins were modulated differently by 28-homoCS and 24-piBL in each cell line. The studied BRs seem to exert potent growth inhibitory and pro-apoptotic effects and could be therefore highly valuable new candidates for prostate anticancer drugs.

1. Introduction

Prostate cancer is a common malignancy and is one of the leading cause of cancer-releated deaths in men worldwide (Jemal et al., 2004). The major cause of the mortality associated with this disease is the metastasis of cancer cells that fail to respond to hormone ablation therapy (Tang and Porter, 1997). As surgery and current chemotherapeutic options seem to be inadequate in curing or controlling prostate cancer, there is a pressing need for the identification of alternative chemopreventive and chemotherapeutic strategies.

Recently, novel phytoestrogens and synthetic estrogens/anti-estrogens/selective estrogen receptor modulators (SERMs) have emerged as promising prostate cancer preventive and treatment agents (Steiner and Raghow, 2003). It is well established that estrogens play important roles in normal growth, differentiation, and development of the prostate (Risbridger et al., 2003). However, any significant contribution by these steroids to the development of prostatic diseases, including prostate cancer in men and animals, is not yet apparent. Estrogens alone, or in synergism with androgens, are potent inducers of aberrant growth and neoplastic transformation in the prostate (Ho, 2004).

Although the etiology of this disease remains largely unknown, age, ethnicity, family history and steroid hormones appear to play a role (Bosland, 2000). The lower circulating estrogen levels have been noted in Japanese men, who are known to have a low risk of prostate cancer when compared with a higher-risk group, Caucasian-Dutch men (de Jong et al., 1991). Furthermore, the incidence of prostate cancer rises exponentially in elderly men, in whom the ratio of estrogen to androgen may increase by up to 40%. This age-related hormonal shift is due primarily to a decline

Abbreviations: 24-epiBL, 24-epibrassinolid; 28-homoCS, 28-homocastasterone; AR, androgen receptor; BRs, brassinosteroids; CDK, cyclin-dependent kinase; DAPI, 4′-6-diamidino-2-phenylindole; ER-α, estrogen receptor α; ER-β, estrogen receptor β; ERs, estrogen receptors; SERMs, selective estrogen-receptor modulators; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick end labeling.

* Corresponding author at: Laboratory of Molecular Pathology, Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic. Tel.: +420 585 639 541; fax: +420 585 632 966.
E-mail address: jana.steigerova@seznam.cz (J. Steigerová).

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2012.08.031

in testicular function and an increase in aromatization of adrenal androgens by peripheral adipose tissue during aging (Gray et al., 1991; Griffiths, 2000).

The androgen receptor (AR) plays a critical role in the development and progression of prostate cancer. AR belongs to the superfamily of nuclear hormone receptors and shows a similarity to progesterone and glucocorticoid receptors. These receptors are composed of well conserved DNA and ligand binding domains, and a less conserved N-terminal region, and they share the same response element on DNA. AR is expressed in the overwhelming majority of prostate cancers, including metastases in patients in whom endocrine therapy has failed. This contrasts with findings obtained in several cell lines of human and rat origin (Hobisch et al., 1995). These immunohistochemical studies have greatly enhanced research on AR expression and function in advanced prostate carcinoma. In prostate tissue epithelial and stromal cells contain AR. It has been suggested that AR in the stroma is a primary androgenic target. Following hormonal stimulation, AR up-regulates the expression of soluble growth factors that bind to membrane receptors on epithelial cells. Candidate mediators of androgenic action between prostate stroma and epithelium are keratinocyte growth factor and fibroblast growth factor-10. The lack of AR expression in some cell lines and patient material could be explained by methylation of the AR gene promoter, CpG island (Jarrard et al., 1998).

Until recently the effects of estrogens on all tissues, including the prostate, were believed to be mediated by the steroid hormone receptors, estrogen receptor α (ER-α) and estrogen receptor β (ER-β). The two ER subtypes are structurally similar, consisting of the six common domains (A–F) found in all steroid hormone receptors, and DNA-binding domains (DBD), which differs by only three amino acids. In contrast, the N terminal A/B domain of both receptors share only 15.5% homology; the ligand binding domains (LBDs) are 58% homologous; and the F domains are distinctly different (Kuiper et al., 1996; Mosselman et al., 1996; Nilsson and Gustafsson, 2000; Pettersson and Gustafsson, 2001). These structural dissimilarities are thought to reflect functional differences between the two receptors. ER-α and ER-β have been shown to bind to the same ligand with different affinities (Kuiper et al., 1997). For example, genistein and some SERMs are known to bind ER-β with higher affinities than to ER-α.

The precise biological functions of the two ER subtypes in the prostate are currently undefined and their possible role in prostate cancer initiation and progression is likewise unknown. Several studies have described the expression of the receptors, at both mRNA and protein levels, in the epithelial and stromal compartments of both normal and malignant adult human prostate glands (Royuela et al., 2001; Torlakovic et al., 2002; Fixemer et al., 2003; Tsurusaki et al., 2003). ER-β is localized predominantly to the basal epithelial cell compartment of the normal human prostate, where ER-α is rarely found. ER-α is mainly expressed in the stroma of the normal gland. Results from these studies suggest that ER-β may exert a protective effect against aberrant cell proliferation and/or carcinogenesis (Chang and Prins, 1999; Poelzl et al., 2000; Signoretti and Loda, 2001).

An important group of phytohormones that can function as growth regulators are the brassinosteroids (BRs). These are steroid substances that play important physiological roles in various plant processes, including cell growth, cell differentiation, root and stem elongation, disease resistance, stress tolerance, and senescence (Bajguz and Tretyn, 2003). BRs have been detected in and isolated from pollen, seeds, fruits, leaves, and galls (Khripach et al., 1999). Furthermore, like their animal counterparts, BRs regulate the expression of numerous plant genes, affect the activity of complex metabolic pathways, and contribute to the regulation of cell division and differentiation (Clouse, 2002). Some natural BRs can inhibit the growth of several cancer cell lines at micromolar concentrations, and it was recently found that natural BRs can induce cell growth-inhibitory responses, arrest cells in the G_1 phase of the cell cycle in hormone-sensitive prostate cancer (LNCaP) cell line and in the G_2/M phase of the cell cycle in hormone-insensitive (DU-145) cell line and induce apoptosis in both prostate cancer cell lines without affecting normal cell growth (Malíková et al., 2008).

This study was focused on molecular mechanism of action of BRs in two human prostate cancer cell lines – LNCaP and DU-145. The choice of these two cell lines was based on the fact that LNCaP cells are androgen-responsive and DU-145 cells are androgen-unresponsive (at the time of clinical diagnosis, most prostate cancers present as a mixture of androgen-responsive and androgen-unresponsive cells). Therefore, eliminating both types of cells seems to be an effective approach for the management of prostate cancer via steroid receptors and the cell cycle machinery. In the first set of experiments, we evaluated whether BR treatment imparts antiproliferative effects in human prostate cancer cells.

2. Materials and methods

2.1. Chemicals

28-homoCS and 24-epiBL (Fig. 1A) were obtained from either SciTech or Olchemim Ltd. (Czech Republic). Stock solutions (10 mM) were prepared in dimethylsulfoxide (DMSO) obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin were also purchased from Sigma. For Western blot analysis, the primary antibodies against: AR (H-90), Bax (clone B-9), caspase-6 (clone H-90), caspase-9 (clone F-7), CDK2 (clone D-12), CDK4 (clone DCS-35), CDK6 (clone C-21), ER-β (H-150), mcm-7 (clone DCS 141.2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Bcl-2 (clone 100) from Biogenex (San Ramon, CA); Bcl-X_L (clone H-62), ER-α (clone 6F11) from Novocastra (Newcastle upon Tyne, UK); Bid and caspase-3 (clone Asp175) from Cell Signaling Technology (Danvers, MA, USA); c-myc (clone Ab-2) from Oncogene Research Products (Boston, MA, USA); PARP (clone C-2-10) from Zymed (San Francisco, CA, USA) and α-tubulin (clone DM 1A) from Sigma were obtained. Other chemicals included Mowiol medium obtained from Calbiochem (Fremont, CA, USA) and the Rainbow™ colored markers Rainbow™ (Amersham Biosciences, Vienna, Austria). The goat anti-mouse-fluorescein, goat anti-rabbit-fluorescein, and Texas Red fluorescently-conjugated secondary antibodies were purchased from Jackson Immunoresearch (Malvern, PA, USA). The secondary goat anti-mouse and goat anti-rabbit IgG-horseradish peroxidase-conjugated antibodies were supplied by Santa Cruz Biotechnology and DakoCytomation (Glostrup, Denmark), respectively.

2.2. Cell cultures

Human prostate cancer cell lines LNCaP (wild-type p53) and DU-145 (p53 mutation at codon 274 and 223) were obtained from the American Type Culture Collection. LNCaP cells were cultured in RPMI medium, while DU-145 cells were maintained in DMEM. All media were supplemented with 10% FBS, L-glutamine (250 mg/l), penicillin (100 U/ml), streptomycin (100 mg/l). All cell cultures were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

2.3. TdT-Mediated dUTP nick end labeling (TUNEL) assay

The terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) technique was used to detect apoptotic cells. Using the appropriate media, cells at densities of either 1.6×10^4 cells/cm^2 (LNCaP) or 1.4×10^4 cells/cm^2 (DU-145) were seeded in 60-mm culture dishes with coverslips. Cells were then grown 24 h and treated with either 28-homoCS or 24-epiBL (IC_{50}) for 6, 12, and 24 h. After the selected treatment periods, the cells were washed with phosphate-buffered saline (PBS) and fixed on the coverslips with cold acetone-methanol (1:1, v/v) for 10 min. Apoptosis-induced nuclear DNA fragmentation was then detected by the TUNEL technique using an *in situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommended protocol. Finally, the coverslips were washed in three changes of PBS, incubated with 4′-6-diamidino-2-phenylindole (DAPI, 50 μg/ml; Sigma) for 10 min in the dark, washed in deionized water, and then mounted on glass slides with the hydrophilic Mowiol medium in glycerol-PBS (1:3, v/v) to measure their fluorescence. The cells were then visualized through a BX50F fluorescence microscope (Olympus, Japan), and the treated and control cells were compared.

2.4. Immunofluorescence labeling methods

LNCaP and DU-145 cells were seeded, cultured, treated, and fixed as described for the TUNEL assay. The cells on the coverslips were then labeled with antibodies against AR, ER-α and ER-β for 90 min at room temperature, and then washed with