Cell cycle distribution response of human lymph node prostate carcinoma (LNCaP) cell line to selected diet-derived agents

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ABSTRACT:

Decline in prostate cancer incidence and mortality rates can be achieved with the adoption of healthier lifestyles and chemoprevention. The use of some notable diet-derived agents has been advocated based on cultural inclinations and basic scientific outcomes. However significant advances in chemoprevention can be made chiefly through the comprehensive knowledge of these naturally-sourced compounds. Alterations to the cell cycle mechanism of LNCaP cell lines were investigated for responses to selected doses of curcumin, 3,3'- diindolylmethane (DIM) and epigallocatechin-gallate (EGCG). The flow cytometry technique in combination with propidium iodide (PI) staining was used for the study. Results obtained reveal different levels and occurrences of cell cycle arrest. The under-studied diet-derived agents possess the capabilities of inhibiting prostate cancer growth and survival by inducing cell cycle arrest although at varying degrees, which needs further investigations.

KEYWORDS: PCa, Cell cycle, Curcumin, DIM, EGCG, flow cytometry.
INTRODUCTION

Prostate cancer (PCA) is the second most commonly diagnosed cancer in men globally (Torre et al., 2015). It is a major public health problem in developed nations, and with steady increases in incidence levels, PCa has been reported to be an emerging public health challenge in Sub-Saharan Africa (Haas et al., 2008; Chu et al., 2011; Jalloh et al., 2013). Aging is a major risk factor for prostate cancer, as incidence increases in males above the age of 50. Other risk factors include race, family history, hormones, diet, inflammation, lifestyle and many others. However, the role any of these factors independently has on PCa initiation and progression is poorly understood (Abate-Shen and Shen, 2000; Hsing and Chokkalingam, 2006).

The carcinogenesis of the prostate demonstrates well-defined stages, which are described respectively as the initiation, promotion and progression stages (Johnson et al., 2008). The initiation stage deals with the origination of PCa, which involves the alterations or changes occurring on otherwise normal genes leading to inactivation of tumour suppressor gene (growth inhibitor and DNA repair proteins) and activation of oncogenes (Li et al., 2005; De-Marzo et al., 2007). These changes further deregulate cellular proliferation, cell cycle balance and apoptotic processes (Zeng and Kyprianou, 2005). The accumulations of genetic and epigenetic DNA alterations are frequently observed in many cancers, including PCa (Li et al., 2005). Normal prostate cells exhibit a balance between cell proliferation and apoptosis, but in PCa, there is a loss of that balance, in which cell proliferation outweighs apoptosis and the accumulation of pre-neoplastic cells initiates tumorigenesis (Zeng and Kyprianou, 2005).

For most PCa cases, the stages of carcinogenesis occur slowly and take a long period of time ( Singh and Agarwal, 2006; Johnson et al., 2008). This “slow growing PCA type” provides an opportunity for chemoprevention, which can either slow or halt the development of the disease. Chemoprevention is recognised as an intervention strategy that has the capability to curb the progression of a disease such as prostate cancer, which thus improves the quality of life of patients, reduce morbidity and decrease mortality (Bertram et al., 1987; Maru et al., 2016).

As with all cells, the prostate cell undergoes cell division cycle in four phases- the G1 phase, S phase, G2 phase and M phase. The G0 phase is the non-proliferative phase in the cell cycle, G1 and G2 phases serve as checkpoint controls prior to entering the next phases. DNA synthesis phase occurs in the S phase and the mitotic phase occurs in the M phase (Elledge, 1996; Meenan and Katiyar, 2008). The cell cycle phases are regulated by some protein kinases known as the cyclin-dependent kinases complexes (CDKs), which become activated when bound with regulatory proteins known as Cyclins (Meenan and Katiyar, 2008). The Androgen Receptor (AR) pathway in conjunction with other signaling pathways such as the MAPK and the PI3K signaling pathways, play very important roles in modulating the cell cycle mechanism in the prostate epithelial cells. These pathways alter the transcriptional activity of AR, causing an alteration of AR activity, which either up-regulates or down-regulate regulatory proteins that play major roles in cell cycle progression such as Rb (retinoblastoma), cyclin D1, cyclin E, CDK6 and CDK1 (Balk and Knudsen, 2008; Pecorino, 2008).

Of interest are three diet-derived agents, namely: Curcumin otherwise known as diferuloyl methane, 3,3'- diindolylmethane (DIM) and epigallocatechin-gallate (EGCG). Curcumin is the major natural polyphenol obtained from the rhizome known as turmeric or Curcuma longa or Zingiberaceae. Curcumin is a common feature in Asian cuisine and is widely used as a spice (Aggarwal et al., 2003; Thangapazham et al., 2006). 3,3'- Diindolylmethane (DIM) is a phytochemical obtained from cruciferous vegetables (Howells et al., 2007). Cruciferous vegetables include brussel sprouts, broccoli and cabbage. Lastly, Epigallocatechin-gallate (EGCG) is the main active polyphenolic compound obtained from both green tea and black tea (Chen and Dou, 2008). This compound makes up approximately 30 - 42% of the total dry weight of green tea (Khan and Mukhtar, 2008).

The anti-proliferative potential of curcumin, DIM, EGCG and their paired combinations on the LNCaP prostate cancer cellular model have previously been investigated (Ibeawuchi-Onuoha, 2016). However, it is highly imperative that these diet-derived are further investigated to understand how growth and survival of LNCaP cells were inhibited in vitro.

In this study, the cell cycle mechanism of LNCaP cell-lines in response to treatments of the selected diet-derived agents will be evaluated by propidium iodide (PI) staining and flow cytometry technique. With a review of previous methodologies and results, the respective IC50 values of curcumin, DIM and EGCG on LNCaP cell lines were revised and adopted from previous studies (Shenouda et al., 2004; Garikapathy et al., 2006; Kimura et al., 2007; Valentini et al., 2009).
MATERIALS AND METHODS

Materials and Reagents

Unless otherwise stated, all reagents were obtained from Sigma (Poole, United Kingdom). Curcumin, DIM and EGCG were obtained from Sigma-Aldrich (Germany).

Cell culture

The human lymph node prostate carcinoma cell lines LNCaP were obtained from American Type Culture Collection (ATCC) (Virginia, USA). The LNCaP cell lines were grown and maintained in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% v/v foetal calf serum (FCS) (Invitrogen, Paisley, United Kingdom). The cell culture media was obtained from Invitrogen (Paisley, United Kingdom) and Sigma-Aldrich (Germany). LNCaP cells were resuscitated from liquid nitrogen by snap-thawing at 37°C, re-suspended in 10 ml pre-warmed medium and centrifuged at 15,000 rpm for 5 minutes. Pellet formed was re-suspended in either 10 ml or 25 ml of fresh media with 10% FCS depending on the culture flasks intended for use. Cells were cultured in a 37°C incubator with 5% circulating CO₂ until the growing cells were approximately 70% confluent. For all protocols, LNCaP cells were always 70% confluent before being washed with PBS, trypsinised and centrifuged. Cell lines were not cultured with antibiotics as they tested negative to any infections. Cells were not used beyond eight passages following their generation.

Cell cycle analysis

LNCaP cells were washed with PBS, trypsinised, centrifuged, re-suspended in sterile warm 10% FCS media (RPMI 1640) and counted. The adjusted LNCaP cell count were seeded into six (6) well plates at 1 x 10⁵ cell density per well and allowed to culture and adhere for 48 hours. After the period of cell adherence, 2 mls of media containing the different treatment concentrations: curcumin (5 µM), DIM (50 µM) and EGCG (30 µM) and the paired combinations of curcumin (5 µM) + DIM (50 µM), curcumin (5 µM) + EGCG (30 µM) and DIM (50 µM) + EGCG (30 µM) were added to cells. Treatments were done in triplicates. These treated cells were left to incubate for 72 hours at 37°C and 5% CO₂.

At the end of the treatment, cells from each well were trypsinised, centrifuged and the supernatant discarded. The treated cell pellets were then re-suspended and fixed in cold 70% ethanol and left at 4°C for 2 hours, this was to ensure that the cell membrane were fully permeable and ready for propidium iodide staining. The treated cells in ethanol were then centrifuged at 400 xg, at 4°C for 10 minutes and the supernatant discarded. The cell residue was then re-suspended in 800 µl of cold PBS, 100 µl of RNase A (1 mg/ml) and 100 µl of propidium iodide (50 µg/ml) and left at 4°C overnight in the dark. The 1 ml content was then transferred into falcon tubes and analysed by the FACScan flow cytometer (BD Biosciences, San Jose, CA) with the FL2 detector and at 488 nm wavelength. Cell cycle analysis was expressed in percentage values of PI-stained DNA in the different cell cycle phases of G₀-G₁, G₂-M and S phases.

RESULTS

To analyse the effect of selected diet-derived agents on LNCaP cell cycle pattern, the cell cycle changes of LNCaP cell were examined and investigated by propidium iodide staining and flow cytometry technique. The cells were treated with curcumin (5 µM), DIM (50 µM) and EGCG (30 µM) and paired combinations of curcumin (5 µM) + DIM (50 µM), curcumin (5 µM) + EGCG (30 µM) and DIM (50 µM) + EGCG (30 µM) for 72 hours. The results shown in Figure 1 represent the alterations in the cell cycle of the LNCaP cell line subsequent to treatment.

Figure 1: Effects of curcumin (C), DIM (D) and EGCG (E) and the paired combinations (C + D), (C + E) and (D + E) on the Cell Cycle profile of LNCaP cell lines following 72 hours treatment. Results shown are means of triplicate values (n = 3) ± standard deviation.

After 72 hours treatment with the respective diet-derived agents, treated cells were compared respectively to untreated and DMSO (control) LNCaP cells. Cell cycle arrest in either the G₁ phase, G₂ – M phase or S phase were ascertained when LNCaP cell numbers were observably higher in the treated test groups (C, D, E, C + D, C + E or D + E) than in the untreated and DMSO (control) groups. In other words, percentage of PI-stained cells in any specific cell cycle...
phase, which is observed to be higher than the positive and negative controls indicated cell cycle arrest.

LNCaP cells treated with DIM (D), curcumin + DIM (C + D) and DIM + EGCG (D + E) respectively were observed to show a G0 - G1 cell cycle arrest. This is because the treatment groups: DIM (D), curcumin + DIM (C + D) and DIM + EGCG (D + E) respectively showed an increased accumulation of cells in the G1 phase when compared to the DMSO (control) and untreated LNCaP cell groups.

The LNCaP cells treatments with curcumin (C) and DIM (D) respectively exhibited G2 - M phase cell cycle arrest, while LNCaP cells treated with EGCG (E) and curcumin + EGCG (C + E) respectively were observed to exhibit S phase cell cycle arrest. Figure 2 below shows the effect of curcumin, DIM and EGCG on the cell cycle distribution of LNCaP cells for a single analysed case after 72 hour treatment.

**DISCUSSION**

From reports, a third of all cancer mortality cases may have been preventable through the increased intake of a chemopreventive regimen (Lodi et al., 2017). Chemoprevention is recognised as the use of natural or synthetic substances or a combination of both to prevent, impede, reduce or reverse the process of carcinogenesis (Ahmad et al., 1997; Lippman and Lee, 2006; Hernández-Ledesma and Hsieh, 2015). Due to the damaging effects and occasional failings of many chemotherapeutic agents in prostate cancer (Singh and Agarwal, 2006), there is a growing demand for more chemopreventive agents from natural and food sources that possess little or no toxicity (Lippman and Lee, 2006).

Many polyphenols and phytochemicals have been observed to possess anti-carcinogenic properties (Manson et al., 2007). The dietary constituents in focus in this study are curcumin, diindolylmethane (DIM) and epigallocatechin gallate (EGCG). Curcumin is the major constituent of turmeric, while DIM and EGCG are sourced from cruciferous vegetables and green tea respectively. It has been reported that curcumin, DIM and EGCG hold promising potentials as chemopreventive agents of prostate cancer (Sarkar and Li, 2004, Ibeawuchi-Onuoha, 2016). Based on epidemiological and experimental studies, diet-derived agents such as curcumin, DIM and EGCG have notably been targets for chemoprevention of many cancers (Maru et al., 2016).

The efficacy of these diet-derived agents has been investigated with the use of LNCaP cell lines (Ibeawuchi-Onuoha, 2016). The selected diet-derived agents were observed to inhibit the proliferation of LNCaP cell lines. Since these investigated diet-derived agents were found to differentially inhibit LNCaP cell growth and survival (Ibeawuchi-Onuoha, 2016), it is imperative that the mechanism responsible for the observed decrease of LNCaP cell growth is further investigated. These studied diet-derived agents may be able to inhibit LNCaP cell growth through the instigation of cell cycle arrest, activation of the apoptotic pathway or the impediment of malignant transformations (Ahmad et al., 1997).

By utilising the flow cytometry technique to investigate the cell cycle pattern after treatment with the investigated diet-derived agents and their paired combinations, three major observations were made. Firstly, LNCaP cells treated with DIM (D), curcumin + DIM (C + D) and DIM + EGCG (D + E) respectively were observed to show a G1 cell cycle arrest. Secondly, LNCaP cells treated with curcumin (C) and DIM (D) respectively exhibited G2 - M phase cell cycle arrest. Lastly, the LNCaP cells treated with EGCG (E) and curcumin + EGCG (C + E) respectively were observed to exhibit S phase cell cycle arrest.

For cell cycle progression, the CDKs become catalytically competent when bound to the Cyclins. Each checkpoint of the cell cycle is regulated by specific Cyclin-CDK complexes. The cyclin D-CDK4 complex and cyclin D-CDK6 complex drive the cell through the early G1 phase. The cyclin E-CDK2 complex is associated with the later G1 phase. The complexes, cyclin A-CDK1 and cyclin A-CDK2 influence the S phase and M phase, while cyclin B is involved in the G2 phase of the cell cycle (Meeran and Khatriyar, 2008).
From published reports, curcumin is capable of instigating a G1 cell cycle phase arrest through the inhibition of cyclin E in LNCaP cells (Aggarwal et al., 2006; Meeran and Khatiyr, 2008). On the other hand, curcumin was reported to be associated with a G2 – M phase cell cycle arrest dose-dependently (Guo et al., 2013). DIM was associated with the instigation of a G1 cell cycle arrest of LNCaP cells (Chinnakannu et al., 2008), while EGCG treated LNCaP cells exhibited G1 phase cell cycle arrest (Singh, Agarwal, 2006; Meeran and Katiyar, 2008; Chuu et al., 2009).

Observations obtained from this study highlight some deviations from previously published reports. However, the paired combinations of some diet-derived agents such as curcumin + DIM (C + D), DIM + EGCG (D + E), which produced a G1 phase cell cycle arrest, and the curcumin + EGCG (C + E), which produced an S phase cell cycle arrest displayed cell cycle arrest patterns that may be attributed to the combined action of both diet-derived agents in a paired combination. It is recommended that further investigations need to be conducted to provide more evidence on the potential of curcumin, DIM, EGCG and their paired combinations to induce cell cycle arrest.

Furthermore, the investigated polyphenols are known extensively to possess antioxidative properties and are sometimes referred to as antioxidants. These antioxidants such as curcumin, DIM and EGCG scavenge ROS (reactive oxygen species) and in addition, they induce the expression of some key regulatory genes (Surh, 2003; Manson et al., 2007). Therefore, the antioxidative role of the investigated diet-derived agents may be linked to the cell cycle arrest observed in this study. The effect of curcumin, DIM and EGCG and their paired combinations on cell cycle regulatory proteins need to be explored further.

Based on this study’s findings, curcumin, DIM, EGCG and their paired combinations have exhibited some potentials to induce cell cycle arrest in prostate cancer cells and there is the possibility for these diet-derived agents can to be used, combined and modified extensively for chemopreventive uses.

**REFERENCES**


Balk, S.P. & Knudsen, K.E. 2008, "AR, the cell cycle, and prostate cancer", *Nuclear receptor signaling*, vol. 6, pp. e001.


