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The anti-cancer effects of carotenoids and other phytonutrients resides in their combined activity

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ABSTRACT

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases, such as cancer. It is now accepted that the actions of any specific phytonutrient alone do not explain the observed health benefits of diets rich in fruits and vegetables as nutrients that were taken alone in clinical trials did not show consistent preventive effects. The considerable cost and complexity of such clinical trials requires prudent selection of combinations of ingredients rather than single compounds. Indeed, synergistic inhibition of prostate and mammary cancer cell growth was evident when using combinations of low concentrations of various carotenoids or carotenoids with retinoic acid and the active metabolite of vitamin-D. In this study we aimed to develop simple and sensitive in vitro methods which provide information on potent combinations suitable for inclusion in clinical studies for cancer prevention. We, thus, used reporter gene assays of the transcriptional activity of the androgen receptor in hormone-dependent prostate cancer cells and of the electrophile/antioxidant response element (EpRE/ARE) transcription system. We found that combinations of several carotenoids (e.g., lycopene, phytoene and phytofluene), or carotenoids and polyphenols (e.g., carnosic acid and curcumin) and/or other compounds (e.g., vitamin E) synergistically inhibit the androgen receptor activity and activate the EpRE/ARE system. The activation of EpRE/ARE was up to four fold higher than the sum of the activities of the single ingredients, a robust hallmark of synergy. Such combinations can further be tested in the more complex in vivo models and human studies.

Introduction

Diet provides desirable health benefits beyond basic nutrition. Life style and especially dietary habits have been closely linked to the risk of various chronic diseases. Considerable epidemiologic evidence indicates an association between the consumption of fruits and vegetables and reduced incidence of various types of cancer [1,2]. Carotenoids, an important group of dietary phytonutrients possess well-documented cancer-preventive activity [3,4]. For example, epidemiological studies have linked increased consumption of lycopene, the red pigment of tomato with decreased prostate [5,6] and breast [7] cancer risk. These findings are supported by in vitro and in vivo experiments showing reduced proliferation, induced apoptosis and a decrease in the metastatic capacity of prostate cancer cells as a result of lycopene treatment [8–10]. Another group of phytonutrients with well-documented cancer-preventive activity are the polyphenols [11,12]. The polyphenol curcumin, the major yellow pigment in turmeric, which is widely used as an Indian spice, attracts much attention due to its strong anti-inflammatory and anti-cancer effects [13], and the anti-proliferative effect of curcumin in prostate cancer cells has been established [14]. Other phytonutrients from human diet such as omega 3 fatty acids seem to provide a promising therapeutic approach [15].

Periodically, we are presented with a new "magic bullet", a dietary component with a therapeutic or preventive effect that should be consumed in high doses. Nonetheless, while most studies support a protective effect of the consumption of fruits and vegetables, significant associations with single compounds have shown conflicting results [16,17]. In addition, supplementation studies using synthetic or purified single agents did not show beneficial effects [18]. Of note, a substantial number of findings derive from cell based-studies using high concentrations of phytonutrients, which

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are often not physiological. The prevailing view is that "magic bullets" are not found, and the beneficial effect of fruits and vegetables is based on the synergistic effect of several components from whole foods, each of them present in low concentration and their combined activity is responsible for the antioxidant and anti-cancer effect of a diversified diet. Staple diet compositions are profoundly dependent on geographical or sociological factors, and have a marked impact on human health.

A tenable view is that the beneficial effects of phytochemical mixtures present in fruits, vegetables and other dietary components [19] reside, at least partly, on complementary and overlapping mechanisms of action of these nutrients on several cellular pathways. Regulation of transcription is a common mechanism for the chemopreventive activity of various phytonutrients and regulation of gene expression has been found to play a significant role in the effect of phytonutrients on many cellular processes including the antioxidant defense mechanism, cell proliferation and apoptosis and hormone signaling and metabolism [20–22].

Prostate cancer is the most common cancer in men [23]. Steroid hormones, particularly androgens, play a role in the initiation and progression of prostate cancer [24]. The pivotal role of androgens in the development of this malignancy prompted research regarding the potential use of phytonutrients, which could interfere with androgen metabolism or its signaling pathway. Another important mechanism in the prevention of cancer including the prevention of prostate cancer is activation of cellular antioxidant defense mechanisms. These include the activation of the Electrophile/Antioxidant Response Element (EpRE/ARE)[1] transcription system which is responsible for the induction of phase II detoxifying and antioxidant enzymes such as glutathione S-transferase, NAD(P)H: quinone oxidoreductase 1, superoxide dismutase, and heme oxygenase 1.

The effects of a tomato extract containing lycopene and other phytonutrients and fish oil containing omega-3 fatty acids on metabolic pathways in prostate cancer patients were recently examined [25]. In this double-blind randomized, placebo-controlled study performed on non-aggressive prostate cancer patients, cDNA microarray analysis revealed that supplementation of these dietary extracts modulate at least two regulatory pathways which are important for the progression of this malignancy: the androgen and estrogen metabolism and the EpRE/ARE transcription system which regulate the oxidative stress response pathway. In view of the importance and relevance of these two pathways in the prostate, we examined in the current study the effect of phytonutrient combinations (including lycopene and omega-3 fatty acids) on the differential modulation of the Androgen Response Element (AnRE) and the EpRE/ARE transcription system in LNCaP prostate cancer cells. To this end, we utilized the sensitive reporter gene assay system in order to explore the effect of different phytonutrient combinations as a selection tool for assessing the best combinations that could be used for prostate cancer prevention in the clinical setting.

Materials and methods

Materials

Crystalline lycopene preparations, purified from tomato extract (>97%), phytoene + phytofluene – a 1:1 mixture (the indicated concentrations are the sum of both carotenoids), tomato extract LycoMato®, vitamin E (Tocopherol mix) and a 1.5:1 mixture of eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA) omega-3 fatty acids (Quimica Industrial Spes S.A, Chile) were a gift of Lycored Ltd., Beer Sheva, Israel. Astaxanthin, silibinin and all-trans retinoic acid were purchased from Sigma Chemicals (Rehovot, Israel). β-Carotene was a gift of DSM (Basel Switzerland). Crystalline curcumin (>95%) was purchased from Cayman Chemicals (Ann Arbor, MI). Carnosic acid (93–97%) was purchased from Alexis Biochemicals (Switzerland) and tetrahydrofuran (THF), containing 0.025% butylated hydroxytoluene as an antioxidant, was purchased from Aldrich (Milwaukee, WI, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and phenol red free RPMI medium, fetal calf serum (FCS), dextran-coated charcoal-stripped FCS (DCC-FCS), and sodium pyruvate were purchased from Biological Industries (Beth Haemek, Israel). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemicals. Acrodisc syringe filter 0.8/0.2 μm (PALL Corporation, MI, USA). Dihydrotestosterone (DHT) was purchased from Ikapharm, Ramat Gan, Israel. 1α,25-Dihydroxyvitamin D_3 (1,25vitD_3) was a gift from Dr. Andrzej Kutner (Pharmaceutical Research Institute, Warsaw, Poland).

Cell culture and treatment

LNCaP – androgen-responsive, PC-3 and DU-145 – androgen-nonresponsive, human prostate cancer cells and MCF-7 – hormone-dependent human mammary cancer cells were purchased from American Type Culture Collection (Manassas, VA, USA).

LNCaP, PC-3 and DU-145 cells were grown in RPMI containing sodium pyruvate (0.11 mg/ml) and DHT (10^{-9} M). MCF-7 cells were grown in DMEM medium containing insulin (0.6 μg/ml). Human foreskin fibroblasts, provided by Soroka University Medical Center skin bank, Beer Sheva, Israel, were grown in DMEM medium. All culture media were supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), nystatin (12.5 μg/ml), and 10% FCS.

Carotenoids were dissolved in THF, solubilized in cell culture medium and the absorption spectra of the compounds were determined as described previously [26,27]. The absorption spectra were measured in each experiment to evaluate the stability and to calculate the actual concentrations of the carotenoids. Curcumin and silibinin were dissolved in DMSO and added to the culture media at the final concentrations specified in the figure legends. Carnosic acid, vitamin E and 1,25vitD_3 were dissolved in ethanol. EPA/DHA mixture was first diluted 1:1 in ethanol and then 1:500 in culture media to receive the final concentrations specified in the figure legends. The final concentrations of the solvents in both the treated and control cells were 0.5% THF, 0.1% ethanol, and 0.05–0.1% DMSO. The compound vehicles did not affect the measurements performed. All procedures were performed under reduced lighting.

Since LNCaP cells detach easily from tissue culture plate surface, the medium was never completely removed from the wells. Medium containing double concentration of all compounds was prepared and diluted 1:2 in the medium present in each well. Final concentrations in wells are specified in figure legends.

Reporter constructs and expression vectors

4xARE reporter construct was kindly provided by Dr. M. Hannink (University of Missouri-Columbia, Columbia, MO). PSA E4-LUC containing the wild type enhancer of the prostate specific antigen (PSA) human gene (496 bp) includes 6xAnRE and was kindly provided by Dr. H.P. Koeffler (Cedars-Sinai Medical Center, Los Angeles). Renilla luciferase (P-RL-null) expression vectors which served as internal transfection standards were purchased from Promega (Madison, WI, USA).

[1] *Abbreviations used:* AnRE, Androgen Response Element; CI, Combination Index; DHT, dihydrotestosterone; DMSO, dimethyl-sulfoxide; DMEM, Dulbecco's modified Eagle's medium; DCC-FCS, dextran-coated charcoal-stripped FCS; EpRE/ARE, Electrophile/Antioxidant Response Element; EPA/DHA, eicosapentaenoic acid and docosahexaenoic acid; FCS, fetal calf serum; NFkB, nuclear factor-kappa B; PSA, prostate specific antigen; THF, tetrahydrofuran; 1,25vitD_3, 1α,25-dihydroxyvitamin D_3.