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Review

Different effect of beta-carotene on proliferation of prostate cancer cells

Joanna Dulińska[a,*], Dorota Gil[a], Jacek Zagajewski[a], Jadwiga Hartwich[b], Marek Bodzioch[b], Aldona Dembińska-Kieć[b], Thomas Langmann[c], Gerd Schmitz[c], Piotr Laidler[a]

[a]*Institute of Medical Biochemistry, Jagiellonian University Medical College, ul. M. Kopernika 7, 31-034 Kraków, Poland*
[b]*Department of Clinical Biochemistry, Jagiellonian University Medical College, Kraków, Poland*
[c]*Department Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany*

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Abstract

It was shown that high doses of beta-carotene (>30 μM) decrease proliferation of prostate cancer cells in vitro. However, it is rather doubtful whether such concentration of beta-carotene is really accessible at cellular level. We studied the effect of 3 and 10 μM beta-carotene on proliferation and gene expression in LNCaP and PC-3 prostate cancer cell lines. Beta-carotene – more efficiently absorbed from medium by androgen-sensitive LNCaP cells – increased proliferation of LNCaP cells whereas it had weaker effect on PC-3 cells. Initial global analysis of expression of genes in both cell lines treated with 10 μM beta-carotene (Affymetrix HG-U133A) showed remarkable differences in number of responsive genes. Their recognition allows for conclusion that differences between prostate cancer cell lines in response to beta-carotene treatment are due to various androgen sensitivities of LNCaP and PC-3 cells. Detailed analysis of expression of selected genes in beta-carotene treated LNCaP cells at the level of mRNA and protein indicated that the observed increase of proliferation could have been the result of slight induction of a few genes affecting proliferation (c-myc, c-jun) and apoptosis (bcl-2) with no significant effect on major cell cycle control genes (cdk2, RB, E2F-1).

Keywords: Beta-carotene; Prostate cancer; Proliferation; Gene expression; Cell cycle

1. Introduction

Despite the extensive knowledge of the role of beta-carotene in major cellular processes, there are some controversies in respect to its potential effect on cancer progression [1]. Beta-carotene (BC), lycopene, lutein, beta-cryptoxanthin, and alpha-carotene account for more than 90% of circulating carotenoids in the human blood [2]. Beta-carotene is the most important precursor of retinol and other retinoids whereas carotenoids have several other functions, such as radical quenching, antioxidant and anticarcinogenic activities, and regulation of cell proliferation [3].

Carotenoids are stored as retinal esters and are transported by several classes of binding proteins, emphasizing that the majority of cellular retinoids exist as protein-bound molecules. The binding proteins consist of the cellular retinol binding protein (CRBP) and the cellular retinoic acid binding protein (CRABP), which together with the retinoid form a tight hormone-binding protein 'cassette' [4]. Intracellular retinol is converted by retinol dehydrogenase into more active compounds called the retinals or retinoids, e.g., 13-*cis*-retinoic acid (13cRA), 9-*cis*-retinoic acid (RA), and all-*trans*-retinoic acid (ATRA) [5]. Retinoids control the growth, differentiation, and apoptosis of cells during embryonic development and throughout life [6]. Acting similarly to hormones, they affect epidermal cell growth, differentiation, and sebaceous gland activity, and have immunomodulatory and anti-inflammatory properties [7,8].

* Corresponding author. Tel.: +48 12 422 74 00; fax: +48 12 422 32 72.
E-mail addresses: mblitewk@cyf-kr.edu.pl, mblaidle@cyf-kr.edu.pl (J. Dulińska).

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Carotenoids have been found to inhibit the growth of several cancer cell lines including melanoma [9], lung [10], mammary [10], colon [11], and leukemia cancer cells [12]. Furthermore, carotenoid treatment has been reported to cause cell-cycle arrest [13], enhancement of gap junctional communications [14], inhibition of the malignant transformation in C3H/10T1/2 cells [15], induction of apoptosis [16], and differentiation [12]. Together, those studies indicate that carotenoids present in vegetables and fruits may be responsible for potential cancer-preventing action by inhibiting the growth of tumor cells.

Prostate cancer has become the second leading cause of cancer-related death among men in most Western countries [17]. The study on levels of retinol and retinoic acid in normal human prostate, BPH, and carcinoma samples reported a 2.5-fold increase of retinol in BPH as compared with normal or carcinoma tissues [18]. Authors suggested that there might be a more efficient uptake of retinol from the circulation perhaps due to higher level of CRBP in BPH tissue. It is of interest as well that they found significantly lower levels of retinoic acid in carcinoma tissue as compared with either BPH or normal prostate tissue, a finding they suggest – based on the earlier work [19] – that might be related to an increased degradation of retinoic acid, which in turn could be facilitated by increased levels of CRABP.

Epidemiologic studies suggest that consumption of vegetables and fruits reduces the risk of prostate cancer [20,21]. Giovannucci et al. [22] reported that ingestion of tomato-based foods and increased plasma lycopene level were significantly associated with a lower risk of prostate cancer. However, Pastori et al. [23] reported that lycopene at 1 μmol/l in association with 50 μmol/l tocopherol inhibited the proliferation of PC-3 and Du 145 cells, whereas lycopene alone was not a potent inhibitor of prostate cancer cell proliferation. Cook et al. [24] suggested that beta-carotene supplementation might reduce the risk of prostate carcinoma among men with low baseline levels of plasma BC. The chemopreventive effects of carotenoids on prostate cancer have also been indicated in several studies with cell lines. The concentration of more than 30 μM BC significantly reduced the growth of three different prostate cancer cell lines, PC-3, Du 145, LNCaP [25], but it is impossible to observe such high concentration of BC in serum even in the long-time supplemented group. The typical BC concentration in serum is 0.35 ± 0.15 μM and in BC supplemented group its total plasma concentration increased about 10–15-fold to 6.831 ± 2.12 μM [24,26]. In another study, without dietary supplementation, serum BC concentration in humans varied markedly but was typical between 0.25 and 1.0 μM. Following consumption of carotenoid supplements or carotenoid-rich fruits and vegetables, serum BC concentration ranging between 8 and 13 μM can be achieved [21,27].

Prostatic growth and development are known to be under strict androgenic control mediated via androgen receptor system. Androgens are also implicated in prostatic neoplasia (benign prostatic hyperplasia and prostate cancer), but the mechanisms remain poorly understood [28]. An important development in the progression of prostate cancer is that these cells become androgen-insensitive and dependent on paracrine or autocrine growth factors. Cell cycle, proliferation, apoptosis, and steroid metabolism-regulating genes have been shown to be important in the biology of prostate. However, relatively little information concerning native in vivo vitamin A dynamics in human prostate tissue is available and even less is known about how certainly retinoid therapies and interactions might affect native vitamin A stores or the flux of the vitamin through this tissue.

Here, we report on studies of the effect of beta-carotene and retinoids on proliferation and gene expression in two different prostate cancer cell lines—LNCaP which is known to be androgen-sensitive and PC-3 which is androgen-insensitive. The medium concentrations of BC employed in our cell culture study include and exceed the range of serum concentration observed in humans [21,24].

2. Materials and methods

2.1. Cell culture

Studies were carried out on human prostate cancer cell lines obtained from ATCC: LNCaP-FGC-10 (human prostate cancer, lymph node metastasis) and PC-3 (human prostate adenocarcinoma, bone metastasis).

Cells were cultured in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS) (Gibco), 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l $NaHCO_3$, 1% L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Polfa, Poland). Semi-confluent cell cultures (initially seeded with 10^4 cells/well on 96-well plate or 1×10^5 to 5×10^5 cells/75 cm^2 culture dish) were supplemented with (a) BC (3, 10, and 20 μM in 0.25% tetrahydrofuran (THF)/ethanol 1:1), (b) ATRA – all-*trans* retinoic acid (0.3, 1, and 3 μM in 0.03% EtOH), and (c) RA – 9-*cis*-retinoic acid (0.3, 1, and 3 μM in 0.03% Et) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 24, 48, and 72 h. Every 24 h culture medium was replaced by a fresh one.

ATRA and RA were dissolved in ethanol to get 10 mM stock solutions.

FCS was substituted with charcoal-stripped fetal calf serum (SF) if steroid hormones were to be added to medium [29].

2.2. Analysis of purity and stability of beta-carotene

Concentration of BC preparations and their purity and stability (lack of any degradation products) in culture media

were studied using the micro-method originally described by Wertz et al. [30].

All chemicals: acetone, ethanol (EtOH), tetrahydrofuran (THF), *tert*-butylmethylether (tBME), and 2,6-di-*tert*-butyl-*p*-cresol (BHT) were of analytical grade. THF, ethanol, and acetone prior to their use were filtered through the Aluminum Oxide column (Sigma).

2.3. Preparation of tissue culture media supplemented with beta-carotene

Crystalline all-*trans*-beta-carotene was obtained from DSM Nutritional Products, Kaiseraugst, Switzerland. It was dissolved in tetrahydrofuran/ethanol 1:1 solvent mix to obtain a 2 mM stock solution that was further appropriately diluted to 3, 10, and 20 μM concentration with cell culture medium. In order to minimize BC oxidation and degradation, all handlings were performed in dimmed light using small aliquots of BC stock solution fractionated into argon-filled vials. Studies on cells cultured in BC supplemented media were carried out in a working room where light was eliminated; the window panels and lamps were covered with special foil insulating from light during all experiments. Typical HPLC chromatogram of a sample of RPMI 1640 medium supplemented with BC is presented below indicating no contamination of BC used in studies (Fig. 1).

2.4. Uptake of beta-carotene (HPLC)

Respective number of cells was cultured in media supplemented with BC. Culture medium was exchanged for the fresh one every 24 h. At the selected time intervals (24, 48, and 72 h) cells were washed with PBS, detached from plates by trypsinization, pelleted (centrifuged 3 min at 8000×*g*), and frozen (at −80 °C) for later analysis.

Uptake of BC by LNCaP and PC-3 cells incubated for 24–72 h in various media (BC with or without DHT) was analyzed using HPLC assessment of BC in media and cell extracts (Roche, column – C18, 218TP54, Vydac, and solvent – acetonitryl/*tert*-butylmethylether/80 mM ammonium acetate/triethylamine – 73:20:7:0.5) at a constant flow of 1.5 ml/min. The solvent was degassed by sonication. Shimadzu SCL-10AVP instrument (Shimadzu, Kyoto, Japan) with the SPD-10AV detector was set for carotenoids at 450 nm [30].

Extraction procedures were performed as follows:

(A) Cells – To frozen cell pellet in Eppendorf tube, 200 μl of acetone (with 0.025% BHT f.c.) was added. The mix was vortexed for 1 min and dried in the Speedvac centrifuge under argon (50 mbar, 0.5 h). Two-hundred microliters of a "solvent mix" (EtOH/THF/tBME 9:1:5, 0.025% BHT f.c.) was added to the dried pellet of broken cells, vigorously vortexed, and centrifuged for 3 min at 8000×*g*.

(B) Media – Twenty-five microliters of medium was mixed with 225 μl of a "solvent mix" in Eppendorf tube, vortexed for 1 min, and centrifuged at 8000×*g* for 3 min.

(C) Both in cell and media extractions, supernatant was carefully transferred to sealed glass vial and applied in 25 μl aliquots to the HPLC sampler system.

All procedures were carried out under dimmed light and cooled conditions to prevent photodamage to the compounds.

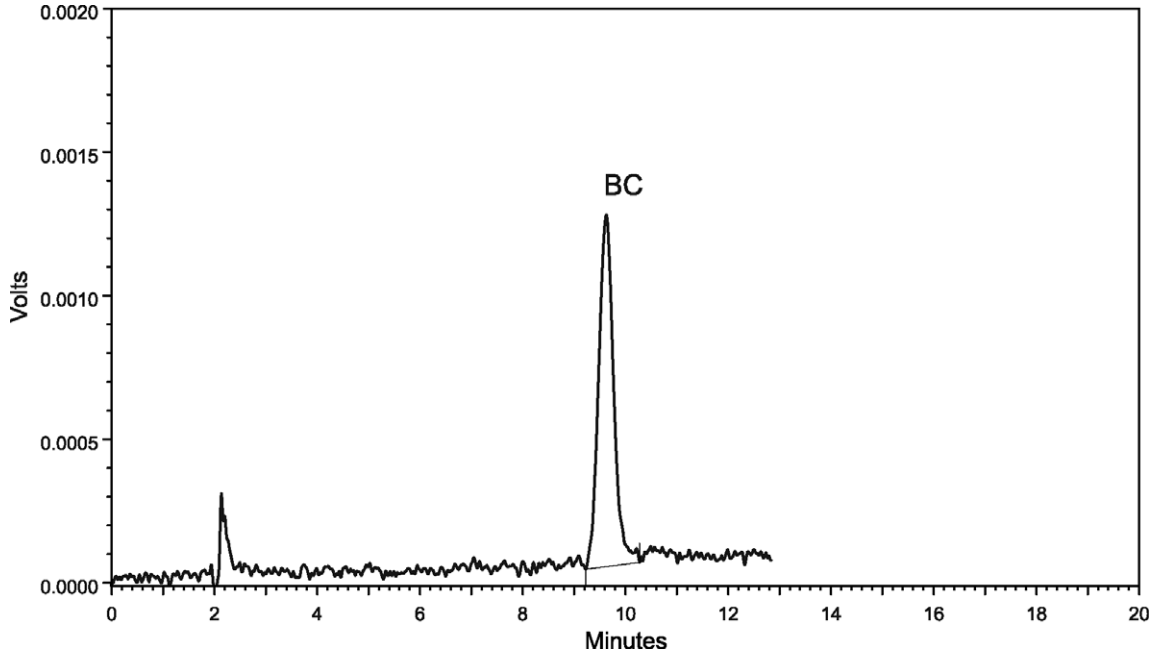

Fig. 1. Typical HPLC chromatogram of culture medium containing beta-carotene (see Materials and methods for details).

Table 1
Primers used for RT-PCR analysis

Gene	Sequence 5′.....3′	Annealing temperature	Cycles
GAPDH	L: 5′-CAC CGC CTC GGC TTG TCA CAT-3′ R: 5′-CTG CTG TCT TGG GTG CAT TGG-3′	59 °C, 30 s	35
c-myc	L: 5′-AAC TGT TCT CGT CGTTTCCGCAA-3′ R: 5′-CAA GAG GCGAAC ACA CaAA CGT CT-3′	55 °C, 60 s	30
c-fos	L: 5′-CAC GAC CAT GAT GT CTC GG-3′ R: 5′-AGT AGA TTG GCA ATC TCG GT-3′	56 °C, 60 s	36
c-jun	L: 5′-TGC AAA GAT GGA AAC GAC CTT-3′ R: 5′-CAG GTT CAG GGT CAT GCT CTG-3′	56 °C, 60 s	36

2.5. Apoptosis test

LNCaP and PC-3 treated or untreated cells were tested with ApoFluo®Green Caspase Activity Assay (Enzyme System Products, ICN Biomedical, Livermore, CA, USA) to detect any effectors phase of apoptosis. Cells of both lines were cultured for 48 or 72 h in the presence of BC dissolved in THF/EtOH (Taxol as positive controls). About 5×10^5 cells were taken for each experiment (two separate experiments). Cells were incubated with the ApoFluo®Green reagent according to the manufacturer's recommendation. Afterwards, cells were harvested, washed to remove any unbound dye, and placed on black microtiter plates. The fluorescence was detected at 520 nm (emission) after excitation at 488 nm.

2.6. Cell proliferation (ELISA-BrdU)

Cells were seeded in triplicates into 96-microwell plates at a density of 1.5×10^3 cells per well and cultured in medium without or with different concentrations of BC (3, 10, or 20 μM), ATRA, and RA (0.3, 1, and 3 μM in 0.03% ethanol) for 1–3 days. ELISA-BrdU (Boehringer-Roche) colorimetric immunoassay test was used for the quantification of cell proliferation. The detection was carried out with the mouse monoclonal anti-BrdU antibody conjugated with peroxidase and tetramethylobenzidine (TMB) as its substrate. The absorbance was measured at 450 nm using an ELISA reader (BIO-TEK Synergy HT-R&D; Microplate Date Analysis Software KC4).

2.7. Cytotoxicity assay (ELISA-LDH)

Cells were seeded in triplicates into 96-microwell plates at density of 1.5×10^3 cells per well and incubated without or with BC (3, 10, or 20 μM) for 24, 48, and 72 h. Afterwards, culture growth media were mixed with the reaction mixture from Cytotoxicity Detection Kit (Boehringer-Roche). The reaction was stopped with 1 M HCl. Colorimetric assay for the quantification of cell death was based on the assay of activity of lactate dehydrogenase (LDH) released from the damaged cells into the supernatant. Absorbance of the colored product – formazane – was measured at 490 nm by ELISA reader (BIO-TEK Synergy HT-R&D; Microplate Date Analysis Software KC4).

2.8. RNA isolation

Following a 48 h incubation with BC (3, 10, and 20 μM) or vehicle alone, cells were harvested and immediately processed to isolate the total RNA. It was purified using the RNeasy Mini Kit from QIAGEN (Germany). The cytoplas-

Table 2
Primers used for real-time PCR analysis

Gene	Sequence 5′.....3′	Annealing temperature (°C)	Cycles
GAPDH	F: CCAGGCGCCAATACGA R: GCCAGCCGAGCCACATC	59	45
Bax	F: GAAGCTGAGCGAGTGTCTAAG R: GTCCACGGCGGCAATC	59	40
BCL-2	F: TGACTGAGTACCT GAACCGGC R: CAGTTCCACAAAGGCATCCC	59	40
CCNA2	F: CAGAAAACCATTGGTCCCTCTT R: ACATGTCCATAGTATGTGGTGAACTCA	59	45
CCND3	F: GCCCTCTGTGCTACAGATTATACCT R: CACTGCAGCCCCAATGCT	59	45
CDK2	F: TTCCCCTCATCAAGAGCTATCTGT R: CCCGATGAGAATGGCAGAA	59	45
RB1	F: AATCCCTTGCATGGC TCTCA R: AGTTGGTCCTTCTCGGTCCTTT	59	45
E2F1	F: CCATCCAGGAAAAGGTGTGAA R: AGCGCTTGGTGGTCAGATTC	59	45

Table 3
The antibody dilution pattern

Protein	The first antibody	Dilution
RB	Mouse monoclonal antibody (4H1) (Cell Signaling)	1:2000
CDK2	Rabbit polyclonal antibody PC44 (Oncogene)	1:4000
C-myc	Mouse monoclonal antibody sc40 (Santa Cruz Biotech.)	1:500
Bel-2	Mouse monoclonal antibody (Pharmingen)	1:2000
Bax	Mouse monoclonal antibody (Pharmingen)	1:2000
Protein	The secondary antibody	Dilution
RB; bcl-2; bax	Anti-mouse goat IgG alkaline phosphatase	1:20 000
Cdk	Anti-rabbit mouse IgG alkaline Phosphatase	1:20 000
c-myc	Goat anti-mouse IgG-HRP	1:15 000

mic protocol was applied (as described in the product manual) to ensure that only mature mRNA species were collected, while genomic mRNA intermediates and DNA had been removed. The yield and purity of isolated RNA were checked by UV spectrophotometric measurement and by electrophoresis in 1.2% agarose gel followed by routine ethidium bromide staining. RNA preparations characterized by A_{260}/A_{280} not less than 1.8 ($\geq$1.8) and showing only 18S (1/3) and 28S (2/3) rRNA bands were used for further analysis. The isolated RNA samples were stored in −80 °C until use for PCR and real-time analysis.

2.9. cDNA synthesis

RNA samples were reverse transcribed in a final volume of 20 μl reaction mixture containing 1× reverse transcriptase buffer – 0.5 mM each dNTP, 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris/HCl (pH 8.3); 10 units of RNase inhibitor (Promega, USA); 10 mM DTT; 50 units of Superscript II RNase H reverse transcriptase (QIAGEN); 1 μM oligo-dT primer (Sigma); and 1 μg total RNA. Prior to addition to reaction mixture the samples of RNA were incubated at 65 °C for 5 min and cooled at 4 °C for 2 min. cDNA synthesis was carried out at 37 °C for 50 min, and reverse transcriptase was inactivated by heating reaction mixture at 93 °C for 5 min.

2.10. Polymerase chain reaction

Amplification of cDNA samples was run in a 20 μl reaction volume that contained the following: 1 μl of synthesized cDNA, 1 μM of each of gene-specific primer pair (Oligo, Poland), 0.5 U Taq DNA polymerase in PCR reaction 1× buffer supplemented with 1.5 mM $MgCl_2$, and 0.2 mM of each dNTPs (QIAGEN). The temperature profile of amplification consisted of activation of *Taq* polymerase at 95 °C for 4 min, denaturation of cDNA at 95 °C for 30 s, elongation at 72 °C for 1 min for the following 29–34

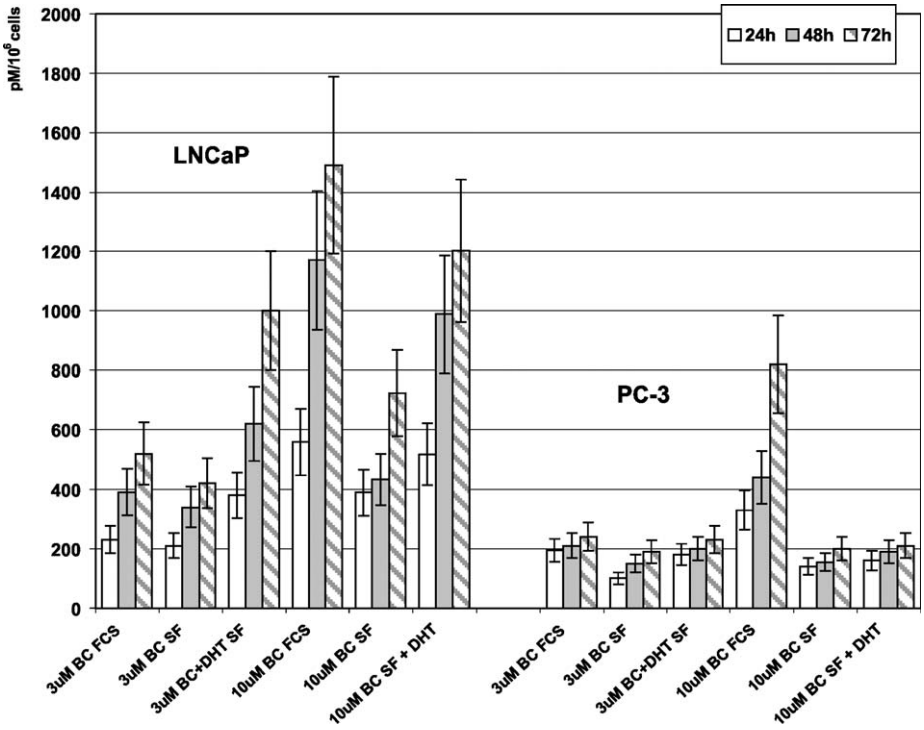

Fig. 2. Uptake of BC by LNCaP (androgen-sensitive) and PC-3 (androgen-insensitive) prostate cancer cells in various media (BC with or without DHT). Experiments were carried out as described under Materials and methods. FCS – fetal calf serum treated as negative reference. FCS was substituted with charcoal-stripped fetal calf serum (SF) if DHT was to be added to medium.

cycles, and finished by an extension step for 10 min. PCR protocols were optimized for each studied gene according to the specific annealing temperature (Table 1). The amplifications were done as single tube PCR in Thermal Cycler PTC 200 (MJ Research).

PCR products were analyzed by 2% agarose gel electrophoresis in TAE buffer (40 mM Tris–acetate, 2 mM EDTA, pH 8.5) and visualized by ethidium bromide staining.

2.11. Microarray expression analysis and RNA extraction

A standard protocol was used for expression analysis in eucaryotic cells, as described in the manufacturer's manual, including hybridization of fragment biotin-labeled cRNA molecules and double phycoerythrin staining. The starting material used per one chip was 10 ng total RNA. The detection of the fluorescent signal was performed by the GeneChi® Scanner 3000 System with the GeneChiπ Operating Software Version 1.0 (Affymetrix, USA).

2.12. Real-time PCR

The relative gene expression analysis was done using the two-step RT-PCR with real-time quantitative amplification performed in the continuous fluorescence detection system-DNA Engine Opticon (MJ Research).

Equal amounts of total RNA (4 μg) were reversed transcribed using the Superscript II Kit (QIAGEN). The amplification was performed with QuantiTect™ SYBR® Green PCR Kit (QIAGEN) using SYBR Green as fluorescent dye. The amplification conditions were as follows: initial incubation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. Subsequently, melting curve analysis was performed to verify the specificity of the PCR products.

The measurements were done in triplicates according to the standard $2^{-\Delta\Delta Ct}$ method, as described by Livak et al. [34] with GAPDH used as a calibrator. All primer pairs were designed using Primer Express 2.0 (Applied Biosynthesis) to work most efficiently in homogeneous PCR conditions. All primer pairs (Table 2) were initially validated passing test for equal amplification efficiencies as described by Livak et al. [34]. Using the presented conditions the amplification efficiency was close to 2. All primer pairs delivered gene single products, which proved to be specific in sequencing.

The detection of the fluorescent signal was performed by the GeneChiπ Scanner 3000 System with the GeneChi®Operating Software Version 1.0 (Affymetrix).

2.13. Western blot analysis

For immunochemical analysis of RB, CDK2, c-myc, bax, and bcl-2, confluent LNCaP and PC-3 cell cultures (~3×10^6–10^7 cells) were rinsed twice with PBS and homogenized on ice three times by sonification, 5 s each (Bandelin Electronic UW 70 Sonopuls Gm 70) in sample buffer—50 mM Tris/HCl (pH 7.5) containing 1 mM EDTA and proteinases inhibitor cocktail (Sigma P-2714). The homogenate was left with 1% Triton X-100 and 0.3% protamine sulfate on ice (1 h) and centrifuged at 16 000×*g* at 4 °C for 1 h (Ultracentrifuge L7-65, Beckman, USA). Protein concentration was determined in supernatants according to Peterson protein assay kit (Sigma P5656) [31]. Fifteen micrograms of total cell extract protein per lane was separated by 8% (RB) or 10% (CDK2, bax, bcl-2, c-myc) SDS-PAGE according to Laemmli [32] and transferred onto PVDF membrane (Roche, Germany). The blotted membrane was treated with 50 mM NaCl/Tris containing 1% bovine serum albumin and 0.1% Tween 20 as blocking agents for 2 h at room temperature and then incubated with recommended dilution of primary antibodies against detected protein (Table 3) for 2 h. After extensive washes, blots were incubated with alkaline phosphatase (AP) conjugated secondary antibodies (bax, bcl-2, Rb, CDK2) or with HRP conjugated secondary antibodies (c-myc) (Table 3). The reaction was detected by the reduction of 4-nitroblue tetrazolium salt in the presence of 5-bromo-4-chloro-3-

Target – transport of retinal				
Title	symbol	No UG	LNCaP 10 uM BC/FCS	PC-3 10 uM BC/FCS
retinol binding protein (plasma membrane) (1)	RBP	Hs.857	1,14	NC
cellular retinal binding protein (2)	CREBP	Hs.270804	1,4	NC
Retinol dehydrogenase (3)	RDH11	Hs.226007	1,4	NC
Retinal dehydrogenase (4)	RDH14	Hs.288880	1,4	NC
Retinoic acid receptor alpha (5)	RARA	Hs.361071	3	NC
Retinoic acid receptor beta (6)	RARB	Hs.436538	1,5	NC
Retinoid X receptor (7)	RXRA	Hs.20084	1	NC

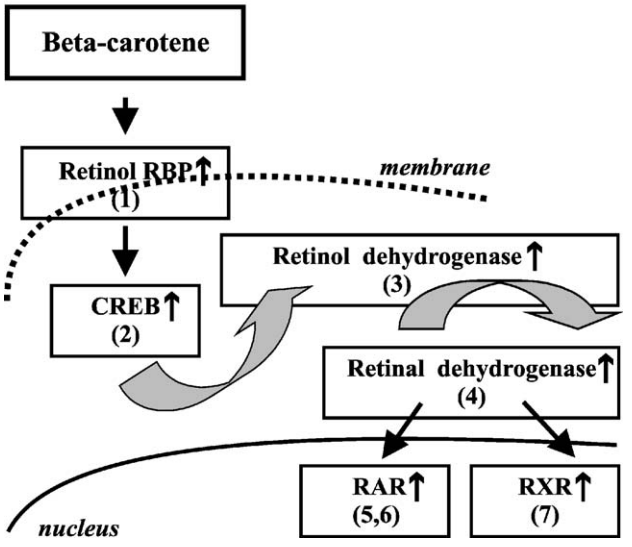

Fig. 3. The effect of BC on the expression of genes coding for its intacellular metabolism.

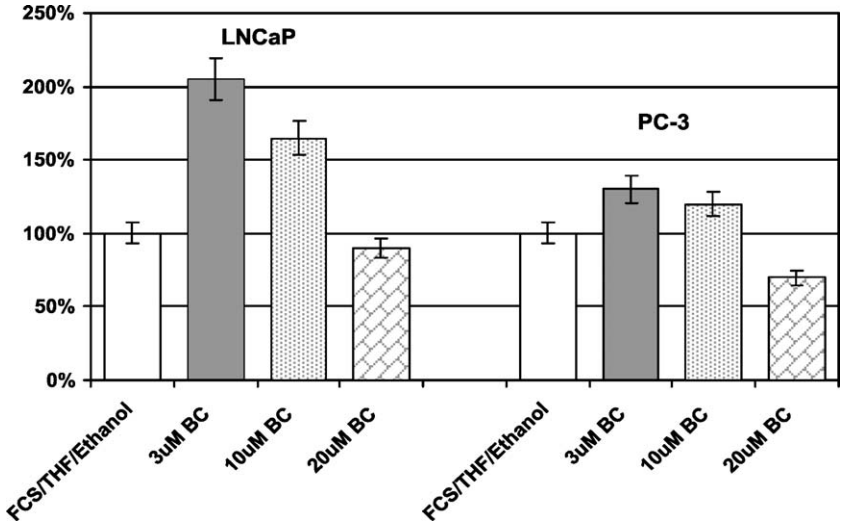

Fig. 4. The effect of 3, 10, or 20 μM BC (THF/ethanol) or vehicle alone on proliferation of LNCaP and PC-3 prostate cancer cell lines after 24–72 h of treatment. Experiments were carried out as described under Materials and methods.

indolyl-phosphate in buffer Tris/HCl (pH 9.5) containing 0.05 M $MgCl_2$ and 1 M NaCl – Roche) or by SuperSignal West Pico chemiluminescent substrate (c-myc). (Pierce).

3. Results

To explore the effect of low concentration of beta-carotene on proliferation and gene expression in prostate cancer cell lines, cells were incubated with either BC (3, 10, and 20 μM) or its derivatives as ATRA and RA (both 0.3, 1, and 3 μM). Routinely, incubation was continued for 24–72 h. No cytotoxic effect of applied concentrations of BC, ATRA, and RA on analyzed cell lines was observed.

Quantitation of amount of BC and analysis of its purity in medium were performed using the respective HPLC setup (Fig. 1). Cells of the studied lines absorbed BC from media in a line-, dose-, and time-dependent manner, although some kind of saturation effect could have been observed for LNCaP cells that absorbed significantly more BC than PC-3 cells (Fig. 2). The meaningful effect of DHT on the uptake of BC by LNCaP cells suggested that absorption of BC by prostate cells might be steroid-dependent. That would explain a much higher uptake of BC by LNCaP than PC-3 cells (Fig. 2). Microarray analysis revealed an increased expression of genes responsible for the transport of retinals – retinol binding protein (RBP) and cellular retinal binding protein (CREBP) as well

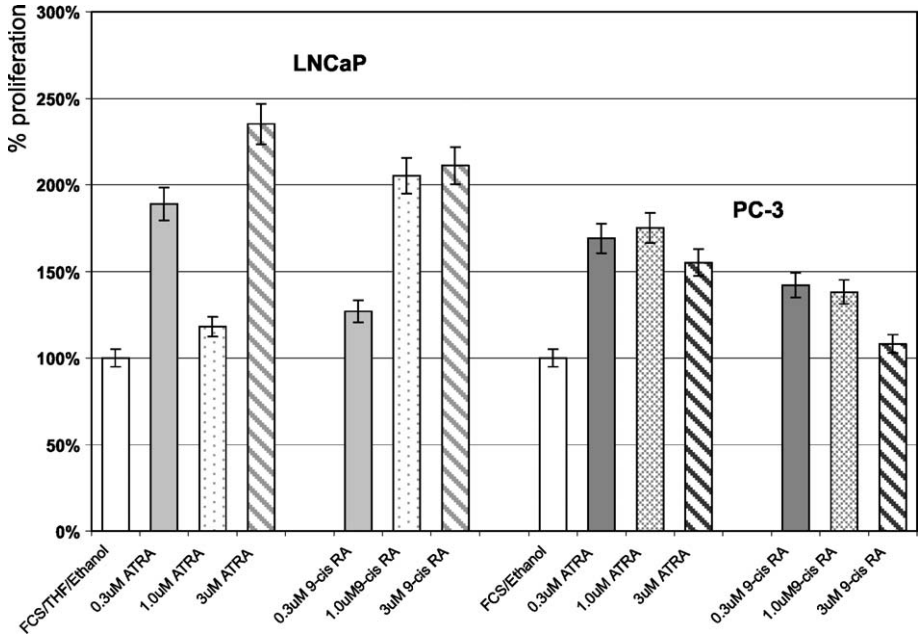

Fig. 5. The effect of 0.3, 1, and 3 μM in 0.03% ethanol ATRA and RA or vehicle alone on proliferation of LNCaP and PC-3 prostate cancer cell lines after 24–72 h of treatment. Experiments were carried out as described under Materials and methods.

Table 4
The group of genes with the strongest response to beta-carotene in the microarray experiments according to the major cellular processes in the prostate cancer cell lines LNCaP and PC-3

Process	Cell line			
	LNCaP, 10 μM BC/FCS 217		PC-3, 10 μM BC/FCS 58	
	Up 121	Down 96	Up 16	Down 42
Signal transduction	14	15	2	3
Cell adhesion	9	2	0	2
Transcription and RNA processing	16	3	1	3
Protein synthesis and growth modification	7	3	1	4
Cell growth and division	6	3	1	0
Cell cycle	7	2	1	4
Intracellular trafficking	2	0	10	20
Steroid metabolism	8	3	0	1
Lipid metabolism	4	6	0	1

as few genes involved in BC metabolism like retinol dehydrogenases and retinoic acid receptors in LNCaP prostate cancer cell line. No such effect was noticed in PC-3 androgen-independent cell line (Fig. 3). The results strongly support the view that absorption of BC by prostate cancer cells is androgen-sensitive.

Prostate cancer cells reacted differently on BC treatment in respect to proliferation—the increase in proliferation of LNCaP cells was observed for 3 and 10 μM BC while there was no effect for 20 μM BC. Incubation of PC-3 cells with 3 and 10 μM BC did not significantly influence the growth of prostate cancer cells but after the treatment with 20 μM BC the meaningful growth inhibition was observed (Fig. 4). ATRA and RA had the similar effect on proliferation of both prostate cancer lines as low concentrations of BC (Fig. 5). These results suggest that retinol is not associated with the inhibitory effects of BC on the viability of prostate cancer cells. Increased proliferation on LNCaP cells upon the treatment with 3 or 10 μM BC may be due to its conversion to retinol leading to synthesis of ATRA or RA in both cell lines. This view is strongly supported by the analogous effect of these BC derivatives on proliferation of both LNCaP and PC-3 cells (Fig. 5). On the contrary, the inhibitory effect of 20 μM BC on proliferation of PC-3 cells and the lack of effect on LNCaP cells may be caused by its–as yet not identified–oxidized forms. Perhaps higher doses of BC lead to DNA damage and in consequence cell cycle arrest or trigger another so far unknown mechanism [25].

Global microarray analysis of genes' expression in LNCaP and PC-3 cells treated with 10 μM BC for 48 h revealed significant differences between both lines in the overall number of up- and down-regulated genes. The response of both lines to BC treatment was not very profound but in the case of androgen-sensitive LNCaP cells the number of genes of which expression changed upon incubation with BC was much higher than in PC-3 cells.

On the effect of BC treatment, genes representing many different cellular activities underwent modulation of their expression. However, when we restricted the analysis and included only genes with the expression changes of at least 0.5 in the logarithmic scale, an interesting pattern of regulation became quite evident. Genes with the highest magnitude of change could be ascribed to one of the following classes: signal transduction, cell adhesion, transcription and RNA processing, protein synthesis and modification, cell growth and division, cell cycle, proliferation and apoptosis, lipid metabolism, intracellular trafficking, and what is very important, steroid metabolism (Table 4).

The focus was put on major genes involved in signal transduction, proliferation, and apoptosis that changed

Table 5
Expression (Affymetrix) of selected genes in LNCaP and PC-3 prostate cancer cell lines treated for 48 h with 10 μM BC

Title	Symbol	Unigene	LNCaP, 10 μM BC/FCS	PC-3, 10 μM BC/FCS
Bcl-2	Bcl-2	Hs.305890	1.4	NC
Bcl-2-associated protein	Bax	Hs.159428	NC	NC
c-jun	JUN	Hs.78465	2.5	NC
c-myc	MYC	Hs.202453	1.7	NC
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	Hs.442398	2.3	NC
Cell division	CDC2	Hs.334562	1.0	1.5
Cyclin A2	CCNA2	Hs.85137	−1.0	−1.0
Cyclin E2	CCNE2	Hs.30462	NC	1.0
Cyclin-dependent kinase	CDK2	Hs.19192	1.4	1.0
P21 (CDKN1A)-activated kinase 2	PAK2	Hs.284275	−1.2	NC
Cyclin-dependent kinase inhibitor 1A (p27)	CDKN1B	Hs.238990	−1.1	NC
E2F transcript factor	E2F	Hs.96055	1.5	NC
Retinoblastoma	RB	Hs.75770	1.1	NC
Cyclin D1	CCD1	Hs.371468	1.3	1.0
Cyclin D3	CD3	Hs.83173	1.5	NC

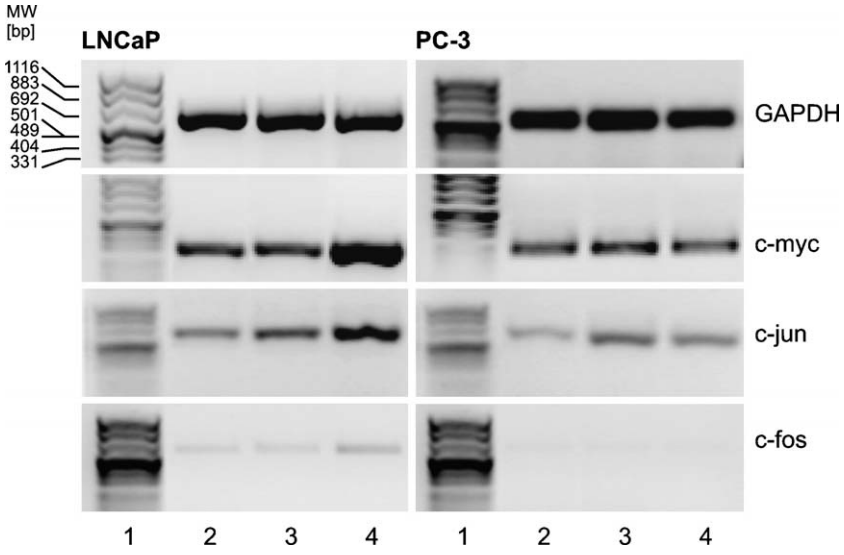

Fig. 6. RT-PCR analysis of c-myc, c-jun, and c-fos transcripts in human prostate cancer LNCaP and PC-3 cell lines. The amplification was carried out for 30 cycles (c-myc), 36 cycles (c-jun, c-fos), or 35 cycles (GAPDH) at temperature programmed as described under Materials and methods and specified in Table 1. The lines show the transcripts from cells cultured in various conditions; lanes 1: standard of DNA, lanes 2: LNCaP or PC-3 10% FCS, lanes 3: LNCaP or PC-3 THF/ethanol, lanes 4, LNCaP or PC-3 10 μM BC. The upper lines show the expression of the housekeeping gene GAPDH at the same conditions.

expression as an effect of BC treatment. Careful analysis of microarray data allowed to select a limited number of genes that were apparently affected by BC in LNCaP cells but remained without change in PC-3 cells (Table 5). The special attention should be paid to c-myc, MYC-associated zinc finger protein, and c-jun as well as a few genes that control the cell cycle (Table 5).

The attempts were performed to detect c-jun, c-myc, and c-fos at the mRNA level (RT-PCR) and to identify the proteins in extracts of LNCaP and PC-3 cells (Western blot) to confirm the observed changes in gene expression revealed by microarray analysis. The analysis disclosed a significant increase of c-myc both at mRNA and protein levels and of c-jun at mRNA level (Figs. 6 and 8). A separate analysis of major genes that control the cell cycle – cyclins A2 and D3, cyclic dependent kinase CDK2, RB1 protein, and E2F1 transcription factor – as well as two constitutive genes involved in mitochondrial apoptotic pathway – Bcl-2 and Bax – was carried out. Using real-time PCR for quantification of mRNA expression and Western blot for protein detection, it was shown that changes in the expression of the noticed genes based on microarray analysis were in fact insignificant. Changes in mRNA expression and consequently in protein synthesis

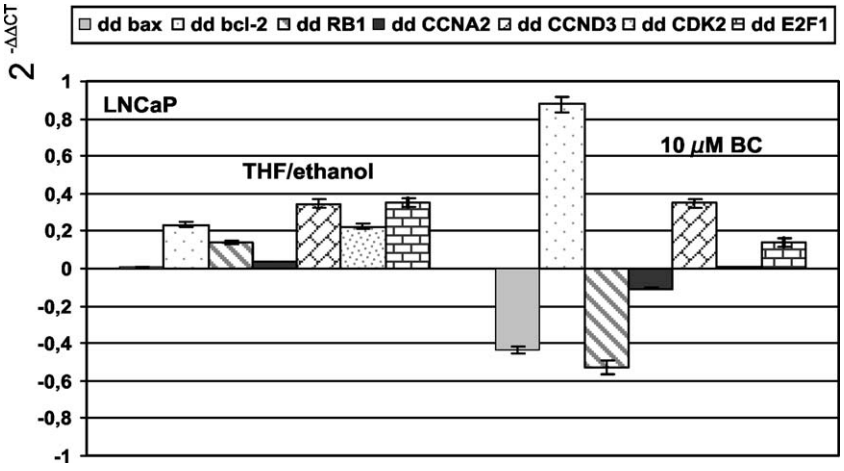

Fig. 7. Real-time PCR analysis of bax, bcl-2, RB, CCNA2, CCND3, CDK2, and E2F1 transcripts in LNCaP and PC-3 prostate cancer cell lines. The total RNA samples were prepared from untreated cell lines and from treated cells with 10 μM BC (48 h). Equal amounts of total RNA (400 ng) were reverse transcribed. The amplification conditions were as follows: initial incubation at 95 °C for 15 min, followed by 40 or 45 cycles of denaturation at 94 °C for 15 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. Subsequently, melting curve analysis was performed to verify the specificity of the PCR products. The measurements were done in triplicates (each three times) according to the standard $2^{-\Delta\Delta Ct}$ method with GAPDH used as a calibrator. All primer pairs were initially validated by a passing test for equal amplification efficiencies. Using the presented conditions the amplification efficiency was close to 2. All primer pairs delivered gene single products, which proved to be specific in sequencing.

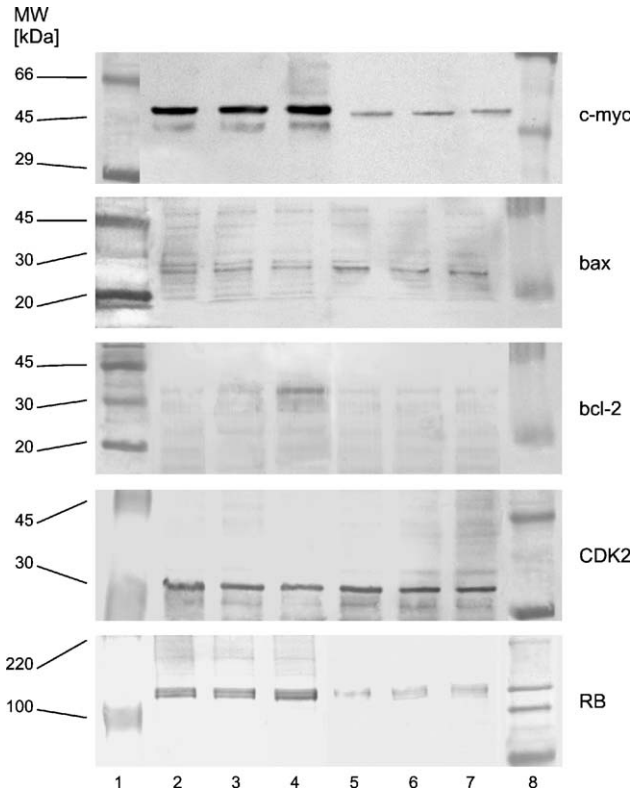

Fig. 8. Immunochemical identification of c-myc, bax, bcl-2, RB1, and CDK2 proteins in whole cell extracts of LNCaP and PC-3 prostate cancer cells cultured by Western blot analysis (see Materials and methods for details). The samples were prepared from untreated cell lines LNCaP and PC-3 (lanes 2 and 5, respectively), LNCaP and PC-3 cells treated (48 h) with THT/ethanol (lanes 3 and 6), and LNCaP and PC-3 cells treated (48 h) with 10 μM BC (lanes 4 and 7). Samples were probed with antibody (c-myc ~47 kDa, bax ~21 kDa, bcl-2 ~29 kDa, CDK2 ~33 kDa; RB position ~110 kDa). The position of the kDa marker protein is indicated (lanes 1 and 8).

were observed only for bcl-2 gene (Figs. 7 and 8). We also noticed a decreased activity of caspases in the apoptotic test after treating LNCaP cells with 10 μM BC (data not shown).

4. Discussion

Results of the numerous reports on the level of beta-carotene in human serum clearly indicated that its average concentration in non-supplemented individuals does not exceed 1 μmol/l and usually is far below that value. After prolonged dietary supplementation of BC, its concentration in serum increases at the best about 10–15-fold to a maximum of ca. 10 μmol/l [21,25,27]. The majority of studies concerning the effect of BC on variety of cells of human organism were performed on much higher concentration of BC in cell culture medium, rarely below 20–30 μmol/l. It seems rather doubtful whether such high doses of BC are really accessible at cellular level.

It is also a well-accepted view that the effectiveness of carotenoids such as BC, e.g., as an antioxidant, is dependent upon its environments. A number of factors may influence metabolism of carotenoids, e.g., concentration, location in the cell, particle or membrane, interaction with other antioxidant, etc., and may result in alterations to its efficacy as an antioxidant [1]. The most striking example is the studies on BC in lung cancer [33]. Beta-carotene may be effective in the prevention of lung cancer if chronically present before or during the phases of initiation and early promotion of the process. Unfortunately BC may also have a co-carcinogenic effect. Supplemental BC is known to inhibit the absorption of the carotenoid lutein which itself may have chemopreventive activity. BC may act as a prooxidant when present in high concentrations in an oxidative environment such as the lungs of smokers in the advanced promotional stage of the neoplastic process. It has been found in the rat lung to cause a booster effect on phase I carcinogen-bioactivating enzymes, including activators of polycyclic aromatic hydrocarbons (PAHs). The mechanism of the possible effect of beta-carotene in enhancing lung cancer in smokers still remains a mystery. The general opinion is that the effect is related to prooxidant activity of beta-carotene or oxidative metabolites of beta-carotene in the context of increased partial pressure of oxygen in smokers' lungs [33].

The effect of BC on lung clearly shows the complexity of its biological activity. With no doubt the final outcome of treatment of various cells with BC strongly depends on the biological status of target. The results of presented studies on the effects of low doses of BC on different prostate cancer cell lines unanimously argue for such view. The uptake of BC – a critical step in its further action – was strongly cell line-dependent. LNCaP prostate cells that are androgen-sensitive absorbed BC from the medium more efficiently than PC-3 androgen-insensitive cells. The results argue for the view that absorption of BC by prostate cancer cells is androgen-sensitive. This should not be a surprise since prostatic growth and development are known to be under strict androgenic control mediated via androgen receptor system. Androgens are also implicated in prostatic neoplasia (benign prostatic hyperplasia and prostate cancer), but the mechanisms remain poorly understood [28,34]. An important development in the progression of prostate cancer is that these cells become androgen-insensitive and dependent on paracrine or autocrine growth factors. Androgens have strong and dose-dependent effects both on proliferation and on differentiation of LNCaP cells. At low concentration, they promote growth; at high concentration, proliferation is reduced but several parameters of secretory function are strongly stimulated [35,36]. However, LNCaP cell proliferation is not only modulated by androgens but also by a number of other ligands acting via nuclear receptors such as VD3, T3, and retinoids. VD3, for example, provokes both stimulatory and inhibitory effects [37].

The effects of BC and retinoic acids – RA and ATRA – on the proliferation of the human prostate cancer cell lines, PC-3 and LNCaP, were quite similar. In cells of both lines, low doses of BC (3 and 10 μM) induced proliferation. The

effect was more profound in LNCaP cell line since it absorbed BC more efficiently leading to its higher concentration in cells. ATRA or RA treatment caused identical response of cells in both lines to low doses of BC. However, higher concentration of BC (20 μM) led to a relative decrease in proliferation in the case of LNCaP cells and a quite remarkable growth inhibition of PC-3 cells.

Williams et al. [25] reported that BC at >30 μmol/l significantly inhibited the growth of PC-3, DU 145, and LNCaP cells. Kotake-Nara et al. [3] presented that alpha-carotene and BC at 20 μmol/l concentration significantly reduced the viability of PC-3 and Du 145 cells but had no effect on LNCaP cells [3], which remains in contrast with our results. However, beta-cryptoxanthin did not reduce cell viability, and most of the non-provitamin A carotenoids at 20 μmol/l reduced the cell viability of PC-3. These results suggest that the provitamin A activity is not necessarily associated with the inhibitory effects on the viability of prostate cancer cells. Carotenoids act not only as antioxidants but also as prooxidants, and the prooxidant action of carotenoids has been suggested to induce apoptosis in tumor cells [38].

So far, there is no clear association between prostate cancer risk and vitamin A intake. Some studies have indicated that a deficiency leads to increased prostate cancer risk, whereas others have suggested that vitamin A supplementation either provides no protection or may lead to an increased risk of prostate cancer [39]. The molecular mechanism underlying the development and progression of prostate cancer is poorly understood. Our study indicates evidence for a possible mechanism by which BC regulates cell growth. It was shown that RA in low concentration increased proliferation of human squamous cell carcinoma lines but higher concentration of RA inhibited cell division [40]. Interestingly, all-*trans* BC enhanced the proliferation and DNA synthesis of BALB/c 3T3 fibroblast cell line induced by a tumor promoter (TPA) [41], which stays in accordance with our results showing that low concentrations of BC as well as its derivatives RA and ATRA significantly increase proliferation of prostate cancer cells. Data here presented provide evidence that low concentrations of BC are efficiently converted to retinol and subsequently to retinoic acids, which leads to increased proliferation of prostate cancer cells, whereas higher concentrations of BC, 20 μM and more, may have anticarcinogenic activity in the case of prostate cancer. The mechanism of this possible anticarcinogenic effect is unclear. We postulate that retinol is not associated with the inhibitory effect of higher concentration of BC on the viability of prostate cancer cells. On the contrary, the inhibitory effect of 20 μM BC on proliferation in both cell lines may be caused by its – as yet not identified – oxidized forms. Perhaps higher doses of BC lead to DNA damage and in consequence cell cycle arrest. Williams et al. [25] observed cell cycle arrest and apoptosis in prostatic cancer cell lines independently with p53 and p21 activities.

Initial global analysis of expression of genes in both types of prostate cancer cells treated with BC performed by means of human high-density microarray showed significant differences in the number of responsive genes and the direction of changes in their expression. In case of LNCaP, the expression of many genes of steroid metabolism was affected while no such effect was noticed in PC-3 androgen-insensitive cells. This indicates strong links between beta-carotene and androgen metabolism in prostate cancer cells. In consequence, it seems that the effect of treatment of prostate cancer cells with beta-carotene may be dictated by their androgen dependence. Control of cell proliferation in the prostate involves a complex interplay between different cell types and steroid hormones, growth factors, and extracellular matrix constituents. Growth factors and steroid hormones activate and may quickly and coordinately modulate the expression of nuclear proto-oncogenes such as c-myc, c-fos, and c-jun, which play a crucial role in the steps that can lead to proliferation and/or differentiation of cells [42].

The detailed analysis of signal transduction proteins and cell cycle control factor genes by RT-PCR technique, real-time PCR, and Western blot revealed the differences between changes of their expression in LNCaP and PC-3 cell lines. The presence of BC in culture medium of LNCaP cells led to increase of expression of c-myc, MYC-associated zinc finger protein, and c-jun genes above its basic level of expression for cells cultured in RPMI 1640 medium containing only 10% FCS. The immunochemical analysis of c-myc in LNCaP and PC-3 whole cell extracts using Western blot method and specific anti-c-myc antibodies clearly showed the increase of c-myc protein in LNCaP cells and no changes of this protein in PC-3 cells, in accordance with the results of detailed analysis of its mRNA. These results might explain why despite the same direction of changes initiated by low doses of BC, the effect of BC on proliferation of androgen-sensitive LNCaP cells is more profound than in the case of PC-3 androgen-insensitive cells. These studies proved that lower doses of BC may have tumor-promoting activity by c-myc-dependent action that might be supported by parallel triggering of steroid metabolism by BC as well as by up-regulation the expression of transcription factors such as c-jun and/or c-fos. The detailed mechanism of this effect remains to be elucidated.

The involvement of retinoid receptors in mediating proapoptotic effects of retinoids is complex due to some retinoids acting in a retinoid receptor-independent manner. However, many studies have suggested a crucial role of RARβ in the modulation of retinoid-induced apoptosis of prostate cancer cells. RARβ is up-regulated during apoptosis induced by the combination of phenylbutyrate and 13-*cis* RA in human and rodent prostate carcinoma cell lines and prostate tumors in the xenograft model [43], suggesting that RARβ expression may mediate the growth-inhibitory effect of retinoids. RARβ was also induced during ATRA-induced

apoptosis of prostate cancer cells [18]. We observed increased expression of both RARα and RARβ upon treatment of LNCaP cells with 10 μM BC. However, this concentration of BC rather led to decreased caspase activity in LNCaP cells, indicating that at relatively low concentration (3 and 10 μM) of BC in medium rather proproliferative and anti-apoptotic signals prevailed. This view is supported by our observation that Bcl-2 protein expression slightly increased upon BC treatment. Therefore, how retinoic acid receptors are possibly involved in apoptosis of prostate cancer cell upon treatment with higher doses of BC requires further studies.

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References

[1] M.D. Xiang-Dong, M.D. Russell, Procarcinogenic and anticarcinogenic effect of beta-carotene, Nutr. Rev. 57 (1999) 263–272.
[2] D.M. Deming, J.W. Erdman, Mammalian carotenoid absorption and metabolism, Pure Appl. Chem. 71 (1999) 2213–2223.
[3] E. Kotake-Nara, M. Kushir, H. Zhang, T. Sugawara, K. Miyashita, A. Nagao, Carotenoids affect Proliferation of human Prostate cancer, J. nutr. 131 (2001) 3303–3306.
[4] S. Nobel, L. Abrahmsen, U. Oppermann, Metabolic conversion as a pre-receptor control mechanism for lipophilic hormones, Eur. J. Biochem. 268 (2001) 4113–4125.
[5] M. Maden, The role of retinoic acid in embryonic and post-embryonic development, Proc. Nutr. Soc. 59 (2000) 65–73.
[6] D.K. Singh, Cancer chemoprevention: Part 1. Retinoids and carotenoids and other classic antioxidants, Oncology 12 (1998) 1643–1658.
[7] R.M. Niles, Recent advances in the use of vitamin A (retinoids) in the prevention and treatment of cancer, Nutrition 16 (2000) 1100–1101.
[8] R.M. Niles, Vitamin A and cancer, Nutrition 16 (2000) 573–576.
[9] M.B. Hazuka, J. Edwards-Prasad, F. Newman, J.J. Kinzie, K.N. Prasad, β-Carotene induces morphological differentiation and decreases adenylate cyclase activity in melanoma cells in culture, J. Am. Coll. Nutr. 9: (1990) 143–149.
[10] J. Levy, E. Bosin, B. Feldman, Y. Giat, A. Miinster, M. Danilenko, Y. Sharonit, Lycopene is a more potent inhibitor of human cancer cell proliferation than either α-carotene or β-carotene, Nutr. Cancer 24 (1995) 257–266.
[11] N. Onogi, M. Okuno, R. Matsushima-Nishiwaki, Y. Fukutomi, H. Moriwaki, Y. Muto, S. Kojima, Antipoliferative effect of carotenoids on human colon cancer cells without conversion to retinoic acid, Nutr. Cancer 32 (1998) 20–24.
[12] H. Amir, M. Karas, J. Giat, M. Danilenko, R. Levy, T. Yermiahu, J. Levy, Y. Sharoni, Lycopene and 1,25-dihydroxyvitamin D3 cooperate in the inhibition of cell cycle progression and induction of differentiation in HL-60 leukemic cells, Nutr. Cancer 33 (1999) 105–112.
[13] L.A. Stivara, M. Savio, S. Quarta, C. Scotti, O. Cazzalini, L. Rossi, L.A. Scovassi, R. Pizzala, R. Melli, L. Bianchi, V. Vannini, E. Prosperi, The antiproliferative effect of β-carotene requires p21waf1/cip1 in normal human fibroblasts, Eur. J. Biochem. 267 (2000) 2290–2296.
[14] L.-X. Zhang, R.V. Cooney, J.S. Bertram, Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action, Carcinogenesis 12 (1991) 2109–2114.
[15] J.S. Bertram, A. Pung, M. Churley, T.J. Kappock IV, L.R. Wilkins, R.V. Cooney, Diverse carotenoids protect against chemically induced neoplastic transformation, Carcinogenesis 12 (1991) 671–678.
[16] E. Nara, H. Hayashi, M. Kotake, K. Miyashita, A. Nagao, Acyclic carotenoids and their oxidation mixtures inhibit the growth of HL-60 human promyelocytic leukemia cells, Nutr. Cancer 39 (2001) 273–283.
[17] A. Jemal, A. Thomas, T. Murray, M. Thun, Cancer statistics, C.A. Cancer J. Clin. 52 (2002) 23–47;
CA Erratum, Cancer J. Clin. 52 (119) (2002) 181–182.
[18] D. Pasquali, C. Thaller, G. Eichele, Abnormal level of retinoic acid in prostate cancer tissues, J. Clin. Endocrinol. 81 (1996) 2186–2191.
[19] J.F. Boylan, L.J. Gudas, The level of CRABP-I expression influences the amounts and types of all-*trans* retinoic acid metabolites in F9 tetracarcinoma stem cells, J. Biol. Chem. 267 (1992) 21486–21491.
[20] E. Giovannucci, S.K. Clinton, Tomatoes, lycopene, and prostate cancer, Proc. Soc. Exp. Biol. Med. 218 (1998) 129–139.
[21] N.R. Cook, M.J. Stampfe, J.E. Manson, F.M. Sacks, J. Buring, C.E. Hennekens, Beta-carotene supplementation of low baseline levels and decreased risk of total and prostate cancer, Cancer 86 (1999) 1783–1792.
[22] E. Giovannucci, E.B. Rimm, G.A. Colditz, et al., A prospective study of dietary fat and risk of prostate cancer, J. Natl. Cancer Inst. 85 (1993) 1571–1579.
[23] M. Pastori, H. Pfander, D. Boscoboinik, A. Azzi, Lycopene in association with α-tocopherol inhibits at physiological concentrations proliferation of prostate carcinoma cells, Biochem. Biophys. Res. Commun. 250 (1998) 582–585.
[24] N. Cook, Effect of beta-carotene supplementation on canver incidence by baseline characteristics in the physicians' health study, CCC, Cancer Causes Control 11 (2000) 617.
[25] A.W. Williams, T.W. Boileau, J.R. Zhou, S.K. Clinton, J.W. Erdman, β-Carotene modulates human prostate cancer cell growth and may undergo intracellular metabolism to retinol, J. Nutr. 130 (2000) 728–732.
[26] Hu. Peifeng, B.D. Reuben, E.M. Crimmins, T.B. Harris, M.H. Huang, T.E. Seeman, The effects of serum beta-carotene concentration and burden of inflammation on all-cause mortality risk in high-functioning older persons: MacArthur studies of successful aging, J. Gerontol., Ser. A, Biol. Sci. Med. Sci. 59 (2004) 849–854.
[27] M.R. Prince, J.K. Frisoli, Beta-carotene accumulation in serum skin, Am. J. Clin. Nutr. 57 (1993) 175–181.
[28] J. Dulińska, P. Laidler, M. Łabędź, Comparative analysis of prostate acid phosphatase and prostate specific-antigen mRNA levels in hyperplastic prostate stimulated with steroid hormones and growth factors, Acta Biochim. Pol. 49 (2) (2002) 357–368.
[29] J. Dulińska, P. Laidler, W.S. Ostrowski, S. Mrozicki, M. Gałka, The effect of dihydrotestosterone on transcription of prostatic acid phosphatase mRNA in human hyperplastic gland, Acta Biochim. Pol. 4 (1997) 751–758.
[30] K. Wertz, N. Seifert, P.B. Hunziker, G. Riss, A. Wyss, C. Lankin, R. Góralczyk, Beta-carotene inhibits UVA-induced matrix metalloproteinase1 and 10 expression in keratinocytes by a singlet oxygen-dependent mechanism, Free Radic. Biol. Med. 37 (2004) 654–670.
[31] L.A. Peterson, A simplification of the protein assay method of Lowry which is more generally applicable, Anal. Biochem. 83 (1977) 346–356.

[32] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[33] R.M. Russell, Beta-carotene and lung cancer, Pure Appl. Chem. 74 (2002) 1461–1467.
[34] S.E. Denmeade, L.J. Sokoll, S. Dalrymple, M. Rosen, A.M. Gady, D. Bruzek, R.M. Ricklis, J. Isaacs, Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models, The Prostate 54 (2003) 249–257.
[35] M.F. Lin, J. DaVolio, R. Garcia, Arenas Expression of human prostatic acid phosphatase activity and the growth of prostate carcinoma cells, Cancer Res. 52 (1992) 4600–4607.
[36] J.V. Swinnen, W. Ulrix, W. Heyns, G. Verhoeven, Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins, Proc. Natl. Acad. Sci. 94 (1997) 12975–12980.
[37] M. Esquenet, J.V. Swinnen, P.P. Van Veldhoven, C. Deneff, W. Heyns, G. Verhoeven, Retinoids stimulate lipid synthesis and accumulation in LNCaP prostatic adenocarcinoma cells, Mol. Cell. Endocrinol. 136 (1997) 37–46.
[38] E. Kotake-Nara, M. Kushiro, H. Zhang, T. Sugawara, K. Miyashita, A. Nagao, Carotenoids affect proliferation of human prostate cancer cells, J. Nutr. 131 (2001) 3303–3306.
[39] A.M. Kamat, D.L. Lamm, Chemoprevention of urological cancer, J. Urol. 161 (1999) 1748–1760.
[40] D.L. Crowe, R. Kim, A.S. Chandrarratna, Retinoic acid differentially regulates cancer cell proliferation via does-dependent modulation of the mitogen-activated protein kinase pathway, Mol. Cancer Res. 1 (2003) 532–540.
[41] Y. Okai, K. Higashi-Okai, Y. Yano, S. Otani, All-*trans* beta-carotene enhances mitogenic responses and ornithine decarboxylase activity of BALB/c 3T3 fibroblast cells induced by tumor promoter and fetal bovine serum but suppressed mutagen dependent umu C gene expression in *Salmonella typhimurium* (TA 1535/pSK 1002), Cancer Lett. 99 (1996) 15–21.
[42] D. Bernard, P. Manzanedo, J. Gil, D.H. Beach, Myc confers androgen-independent prostate cancer cell growth, J. Clin. Invest. 112 (2003) 1724–1731.
[43] R. Pili, M.P. Kruszewski, B.W. Hager, J. Lantz, M.A. Carducci, Combination of phenylbutyrate and 13-*cis* retinoic acid inhibits prostate tumour growth and angiogenesis, Cancer Res. 61 (2001) 1477–1485.