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RESEARCH PAPER

# Angiogenesis in Balb/c mice under beta-carotene supplementation in diet

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**Abstract** Angiogenesis is a process of new blood vessel formation from pre-existing ones. The most important steps in angiogenesis include detachment, proliferation, migration, homing and differentiation of vascular wall cells, which are mainly endothelial cells and their progenitors. The study focused on the effect of beta-carotene (BC) supplementation (12,000 mg/kg) in the diet on angiogenesis in Balb/c mice. Female Balb/c mice were fed for 5 weeks with two different diets: with BC or without BC supplementation. After 4 weeks of feeding, Balb/c mice were injected subcutaneously with two matrigel plugs with or without basic fibroblast growth factor (bFGF). Six days later, the animals were killed, and the matrigel plugs were used for immunohistochemical staining with CD31 antibody and for gene expression analysis. Microarray and Real-Time PCR data showed down-regulation of genes involved in proliferation and up-regulation of genes encoding inhibitors of apoptosis, proteins regulating cell adhesion, matrix-degrading enzymes and proteins involved in the VEGF pathway. The results of this study demonstrated that BC proangiogenic activity (with or without bFGF) in vivo seemed to be more significantly associated with cells' protection from apoptosis and their stimulation of chemotaxis/homing than cell proliferation.

**Keywords** Angiogenesis · Beta-carotene · Chemotaxis · Microarray

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## Introduction

Angiogenesis, the process of new blood vessels formation, plays a central role in both normal (physiological) and pathological events [6]. The most important steps in angiogenesis include detachment, proliferation, migration, homing and differentiation of the endothelial and/or their progenitor cells [14]. Beta-carotene (BC), which represents provitamin A carotenoid obtained in diet from dark green and colourful vegetables, is a source of retinoids that act as ligands for the nuclear retinoid receptors and promote cell differentiation in humans [16]. Our previous in vitro studies confirmed the proangiogenic effect of BC on angiogenesis in human umbilical endothelial cells (HUVECs) [7, 15] and endothelial progenitor cells (EPCs) [9]. Physiological concentrations of BC (3 μM) did not change the tubulogenic activity of HUVECs in the in vitro angiogenesis model, but BC stimulated chemotaxis of both cell types (mean ratio of stimulated migration by BC 3 μM for HUVECs was 4, for EPCs it was 6). These observations were in agreement with the microarray data, confirmed by Real-Time PCR (RT-PCR), which revealed changes in the expression of the genes that influenced cell adhesion, matrix reorganization, homing and chemotaxis. The up-regulated genes included early growth response 1 (EGR1) (cell differentiation activator), baculoviral IAP repeat-containing 3 (BIRC3) (inhibitor of apoptosis), IL8 and chemokine (CC motif) ligand 2 (CCL2) (activators of migration), CXCL12 and CXCR4 (activators of homing) [9, 15]. Thus, our in vitro studies demonstrated that BC's physiological concentrations stimulate early steps of angiogenesis by the activation of cellular migration, matrix reorganization and decrease in cell adhesion. Prochemotactic and homing activity of BC in EPC cells suggested its potential role in the physiology of progenitor cells that might make them useful in therapy directed towards tissue repair [17]. The BC effects observed in the cell culture needed to be confirmed in vivo. Here, we investigated the effects of BC in wild type Balb/c mice using a matrigel angiogenesis model.

## Materials and methods

### Mice

The protocol was accepted by the Jagiellonian University ethics committee. To investigate the effect of BC (12,000 mg/kg in the diet) on angiogenesis, 8 week old female Balb/c mice ($n = 6$–10 per group) were fed with chow diet (Kliba 2415) [Provimi Kliba AG, Switzerland containing vitamin A 1,400 U/kg] supplemented with BC beatlets [12%–1,200 ppm BC] or control, no-BC beatlets (kindly supplied by DSM Neutraceuticals) for 5 weeks. In the control diet (without BC beatlets), amount of vitamin A was the same as in the diet with BC beatlets. To estimate BC intake by animals, BC concentration in serum was controlled after 5 weeks of experimental feeding with the HPLC micromethod developed by DSM neutraceuticals (Roche Vitamins AG) (Kaiseraugust, Switzerland) [2].

### The used model of angiogenesis

Balb/c mice received sterile abdominal injections of 2 × 500 μL matrigel subcutaneously 4 weeks after initialization of their assigned diets. To induce angiogenesis, matrigel plug contained bFGF (50 nM) and for control no-bFGF solvent (phosphate-buffered saline with 0.5% BSA). Six days later, the animals were killed, and the matrigel plugs were removed. The paraffin embedded sections were immunohistochemically stained for CD31 (PECAM) (Becton Dickinson) antigen, a marker characteristic for endothelial cells. Number of capillaries with and without lumen along with the number of separate PECAM positive cells was counted under light microscope in five different fields in each of the three slices taken from different parts of each plug by an uninformed, trained morphologist according to the published protocols [21].

To investigate the effect of local BC injection in an in vivo model of angiogenesis, female Balb/c mice from different group were chosen. The mice were fed a standard lab chow for rodents (containing 3% fat) (GAMRAT, Poland), then injected for 6 days with matrigel containing BC [3 μM] ($n = 5$) with or without bFGF [50 nM] ($n = 5$). Then, mice were killed, matrigel plugs were removed and immunohistochemically stained for CD31 antigen. The angiogenic response was measured as described earlier.

### The microarray analysis

mRNA was obtained from the cells that populated, matrigel plugs were removed from the mice fed diets with or without BC. RNA samples were isolated using Trizol (Invitrogen Life Technologies, USA) and purified with SV Total RNA Isolation System Kit (Promega, USA). The total mRNAs from three mice were pooled together and used as a single sample for a microarray study. The analysis was carried out on a custom-made cDNA microarray chip that contained 3153 unique sequences, 387 representative sequences originating from adipose tissue, 2456 representative sequences originating from large and small intestinal libraries and 298 selected named cDNAs [19]. Array construction, cDNA synthesis, hybridization, scanning, data acquisition and normalization were done as previously described [19]. Only data points that generated

average signals twice as strong as background were used for further analysis. The values of the replicates were averaged and used to calculate fold differences between three treatment groups. Results were presented as a ratio of average signal of a given sample to average control signal that reflected relative values of gene expression. The results from four experiments, which included pooled material from three mice each, were analysed with the student *t*-test to determine statistical significance in observed changes of the gene expression (significance considered at $P < 0.05$). The genes selected for further analysis demonstrated significant differences in their signal intensities ($P < 0.05$) and relative changes in their expression greater than 1.4-fold.

### Real-Time PCR (RT-PCR)

RT-PCR was carried out in order to investigate the regulation of genes, whose expression was significantly altered during the microarray analysis. Our selection included genes that control BC metabolism (Retinoic acid receptor responder (tazarotene induced) 2 (Rarres2) and Chemokine-like receptor 1 (Cmklr1)), code for apoptosis inhibitor (metallothionein1 (Mt-1)), regulate cell homing (R-cadherin (Cdh4)), code for zeta-chain [TCR]-associated protein kinase (Zap 70), tenascin-C (TnC), angiogenesis activators (c-fos induced growth factor (Figf)), secreted acidic cysteine rich glycoprotein (Sparc), selenoprotein N1 (Sepn1) and regulator of Ras/Rho/Rac-signalling pathway DNA segment Chr10 and ERATO Doi 610 (D10Ertd610, Geft). The sequences of the primers used in this study are presented in Table 1. Each RT-PCR was performed in a reaction mixture containing QuantiTect SYBR Green PCR (Qiagen, Germany) mix and primers using the DNA Engine Opticon II system (MJ Research, Germany). The thermal profile of PCR reaction included initial denaturation for 15 min at 95°C, followed by 40 amplification cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C. Following PCR amplification, melting curve analysis was performed with a temperature profile slope of 1°C/s from 35 to 95°C. Results were calculated using the software Calculation Matrix for PCR Efficiency REST-XL (Gene Quantification, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was selected as a reference since its expression did not change in microarray data.

### Statistical analysis

Statistical analysis was performed using one-way ANOVA. All results were expressed as mean ± SEM. Statistical comparisons were made by unpaired *t* tests for comparisons of quantitative variables. $P < 0.05$ was considered significant.

## Results

Serum BC concentrations in the Balb/c female mice fed BC-containing diet ranged 0.2–0.97 μmol/L depending on the amount of food intake (mean 0.69 ± 0.14), whereas no detectable amounts of BC were found in the control mice. Activation of angiogenesis was observed in the matrigel plugs removed from the mice on BC-enriched diet (Fig. 1a, b). Similarly, local injections of BC along with matrigel potentiated bFGF stimulated angiogenic response (Fig. 2a, b). Microarray analysis of gene expression in the cells isolated from the matrigel plugs identified several genes related to regulation of apoptosis, proliferation and adhesion. Analysis of the microarray data revealed BC-mediated (or BC with bFGF) up-regulation of two inhibitors of apoptosis: gelsolin (Gsn) and metallothionein 1(Mt1) (Table 2). The anti-apoptotic activity of gelsolin is related to the prevention of cytochrome c release from the

**Table 1** Sequence of primers for real-time PCR (for mouse sequences)

| Gene symbol | mRNA sequence | Forward primer | Reverse primer |
|---|---|---|---|
| Rares2 | NM_027852 | 5′-TGAGGTGAAGCCATGAAGTG-3′ | 5′-GCTGAGTTCGGGCTCTGT-3 |
| Cmklr1 | NM_008153 | 5′-CAAGGACCAGGACTGGAGTT-3′ | 5′-GATGCCGGAGTCGTTGTAA-3′ |
| Mt1 | NM_013602 | 5′-TCACCAGATCTCGGAATGG-3′ | 5′-AGGTGCACTTGCAGTTCTTG-3′ |
| Sepn1 | NM_029100 | 5′-GACGACCAGTCCTGCTGAG-3′ | 5′-CATTGAGGAGAGTGAGGATGG-3′ |
| Cdh4 | NM_009867 | 5′-GACGTGAGGGACAACATCCT-3′ | 5′-CTGTTGCAGCTGGCTGAG-3′ |
| Zap70 | NM_009539 | 5′-CGGAGTGTCCTCCTGAGAT-3′ | 5′-CGCATACGTTGTTCCACAGT-3′ |
| Tnc | NM_011607 | 5′-ACCGCAGAGAAGAATTTTGG-3′ | 5′-TCCCCATGGTCTTGTAGGTC-3′ |
| Figf | NM_010216 | 5′-TTTTGCAAGACGAGACTCCA-3′ | 5′-GCGATCTTCATCAAACGTCA-3′ |
| Sparc | NM_009242 | 5′-AAGTACATTGCCCTGGAGGA-3′ | 5′-AGCAGGAGGCGTGAACTTAG-3′ |
| Geft | NM_028027 | 5′-AGGTGTCCAGAGTGCTGTTG-3′ | 5′-GGAAGGGCTGGAGAGTCAT-3′ |
| Gapdh | NM_001001303 | 5′-TCACCACCATGGAGAAGGC-3′ | 5′-ACACCCATCACAAACATGG-3′ |

mitochondria into the cytosol [8], while metallothionein 1 is known to protect against toxic metal and oxidative stress [1]. Among genes related to proliferation, the activator of S phase kinase gene expression (Ask) was significantly up-regulated by BC. The Ask kinase blocks cell-cycle transition by the inhibition of Cdc7/Dbf4 protein kinase activity, and the subsequent failure of Cdc45 binding to chromatin occurs. Combination of BC with bFGF resulted in a significant up-regulation of Mark3, an inhibitor of G2/M Cdc25C-associated kinase 1. The microarray data also demonstrated changes in the expression of several adhesion-related genes (Table 2), which were also listed previously [4]. BC (or BC with bFGF) also caused down-regulation of several genes encoding collagens (Col5a2, Col6a2, Col1a2, Col24a1) and up-regulation of genes encoding extracellular matrix-degrading enzymes described previously [5]. BC in conjunction with bFGF led to up-regulation of genes' expression in the VEGF pathway (tenascin-C (TnC), periostin (Postn), c-fos (Figf), osteoblast specific factor (Mif)). VEGF is a known inducer of cellular proliferation, migration and tube formation in matrigel and thus stimulates neovascularization [12]. BC (or BC with bFGF) also influenced expression of genes coding for proteins participating in the other intracellular-signalling pathways involving Ras proteins (Table 2).

Real-time PCR was performed to confirm BC-mediated regulation of gene expression related to BC metabolism (Rarres2-receptor, Chemokine-like receptor 1 (Cmklr1)) and vascular network formation such as regulation of progenitor cells homing, angiogenesis dependent on VEGF pathway activity and control of Ras/Rho/Rac-signalling pathway that can influence cell chemotaxis [10]. Our Real-

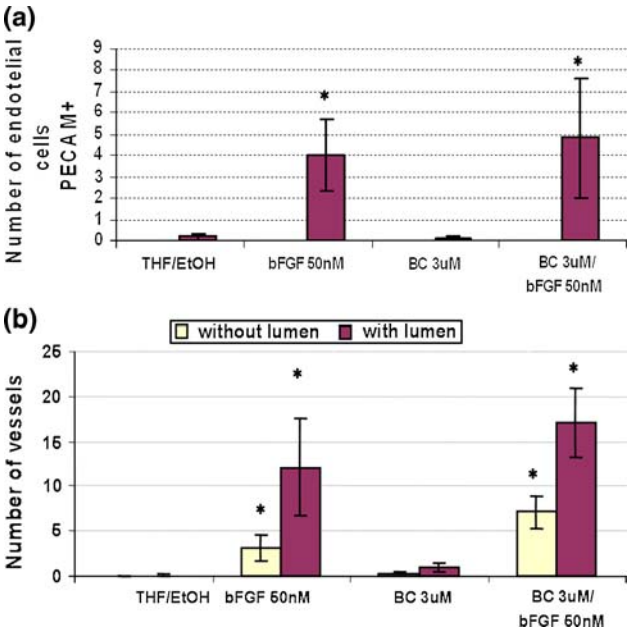



**Fig. 2** Effects of BC [3 μM] injected locally subcutaneously with matrigel mixed with or without bFGF [50 nM] to mice Balb/c. **a** Number of single endothelial cells migrated into matrigel. **b** Number of vessels with lumen (*red*) and without lumen (*yellow*) in matrigel plug. Values are expressed as mean ± SD, significance $P < 0.05$ ($n = 5$)

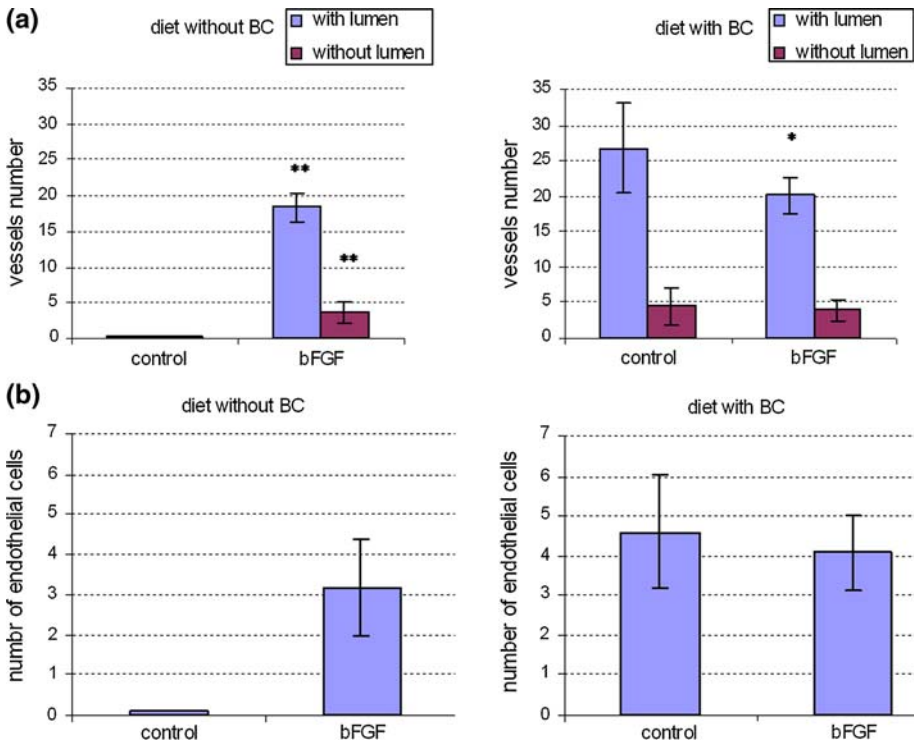
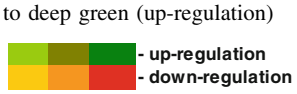



**Fig. 1** Effects of BC in diet on angiogenesis in mice Balb/c. **a** Number of vessels with lumen (*blue*) and without lumen (*red*) in matrigel plug. **b** Number of endothelial CD31 (PECAM)-positive cells penetrating the matrigel plugs. Values are expressed as mean ± SD, significance $P < 0.05$ ($n = 5$–6 animals)

**Table 2** Microarray analysis of selected genes regulated by BC in cells harvested from matrigel in angiogenesis model in vivo

| | bFGF | BC | BC + bFGF |
|---|---|---|---|
| **regulation of proliferation** | | | |
| Cdc42 | 2,28 | 1,05 | 1,82 |
| Mif | 1,85 | NC | 2,36 |
| Tnfsf13 | NC | NC | 2,28 |
| **regulation of S phase** | | | |
| Ask | NC | 3,63 | NC |
| Foxg1 | 2,16 | NC | NC |
| Figf | NC | NC | 3,22 |
| **M phase** | | | |
| Calr | 2,71 | 1,33 | 1,47 |
| Mlh3 | NC | NC | 6,59 |
| dmr-n9 | -1,88 | -2,02 | -1,40 |
| **regulation of M phase** | | | |
| Anapc5 | -3,33 | -3,61 | -3,70 |
| **G2/M** | | | |
| Mark3 | NC | NC | 3,23 |
| **apoptosis** | | | |
| Gsn | 4,99 | 6,17 | 2,16 |
| Mt1 | 5,05 | 2,06 | 6,52 |
| Lzp-s | 2,63 | 1,82 | 3,54 |
| Tde1 | 2,12 | NC | NC |
| Ptprz1 | 3,69 | NC | NC |
| Mif | 1,85 | NC | 2,36 |
| **cell-cell adhesion** | | | |
| Cdh4 | NC | 4,79 | NC |
| Pcdha12 | NC | 2,00 | NC |
| **cell-extracellular matrix adhesion** | | | |
| Col5a2 | 1,37 | 1,15 | 2,02 |
| Col6a2 | 2,02 | NC | NC |
| Col1a2 | 2,61 | 1,50 | 4,11 |
| Col24a1 | -4,38 | -5,40 | -6,80 |
| Tbl2 | -2,96 | -2,31 | -1,64 |
| Lzp-s | 2,63 | 1,82 | 3,54 |
| Sparc | 2,35 | 1,51 | 2,89 |
| Dcn | 2,03 | 1,27 | 2,19 |
| Retnla | -2,53 | 1,19 | -4,43 |
| Postn | NC | 1,87 | 2,37 |
| Tde1 | 2,12 | NC | NC |
| Zp3r | NC | NC | 2,59 |
| Stim1 | NC | NC | 2,35 |
| **extracellular protease** | | | |
| Adam5 | NC | 2,19 | NC |
| Timp1 | 4,57 | NC | 4,89 |
| Tnc | 1,61 | NC | 2,25 |
| **migration** | | | |
| Gsn | 4,99 | 6,17 | 2,16 |
| Cdh4 | NC | 4,79 | NC |
| Runx3 | 1,32 | 2,17 | NC |
| D10Ertd610e/GEFT | NC | NC | 2,65 |
| Postn | NC | 1,87 | 2,37 |
| Sparc | 2,35 | 1,51 | 2,89 |
| Arfip2 | NC | NC | 3,20 |
| Figf | NC | NC | 3,22 |
| Foxg1 | 2,16 | NC | NC |
| Tnc | 1,61 | NC | 2,25 |
| Prrx2 | 2,07 | NC | NC |
| Mark3 | NC | NC | 3,23 |
| **GPCR receptor pathway** | | | |
| Cmklr1 | NC | 4,45 | NC |
| Zap70 | NC | 2,00 | NC |
| Capn3 | 3,00 | 1,27 | NC |
| **VEGF pathway** | | | |
| Tnc | 1,61 | NC | 2,25 |
| Postn | NC | 1,87 | 2,37 |
| Figf | NC | NC | 3,22 |
| Mif | 1,85 | NC | 2,36 |
| Dapp1 | NC | 2,94 | NC |
| Cdc42 | 2,28 | 1,05 | 1,82 |
| **small GTPase mediated signal transduction** | | | |
| Arl2 | NC | NC | 2,61 |
| Rit2 | NC | NC | 2,30 |
| Arfip2 | NC | NC | 3,20 |
| D10Ertd610e/GEFT | NC | NC | 2,65 |
| **Ras/Mapk pathway** | | | |
| Sepn1 | -13,15 | -16,24 | -10,11 |
| Map4k6 | 4,06 | NC | NC |

Investigated conditions included bFGF, bFGF-diet without BC versus control diet without BC; BC, control diet with BC versus control diet without BC and bFGF + BC, bFGF-diet with BC versus control diet without BC. Colour intensities of boxes corresponding to each gene reflect the degree of change in their expression. The colours range from either light orange to red (down-regulation), or light to deep green (up-regulation)




Time PCR results (Fig. 3) confirmed the microarray data that indicated up-regulation of Cmklr1, metallothionein1 (Mt-1), an inhibitor of apoptosis, genes related to homing such as R-cadherin (Cdh4) and tenascin-C (TnC), angiogenesis activators that included c-fos induced growth factor (Figf), secreted acidic cysteine rich glycoprotein

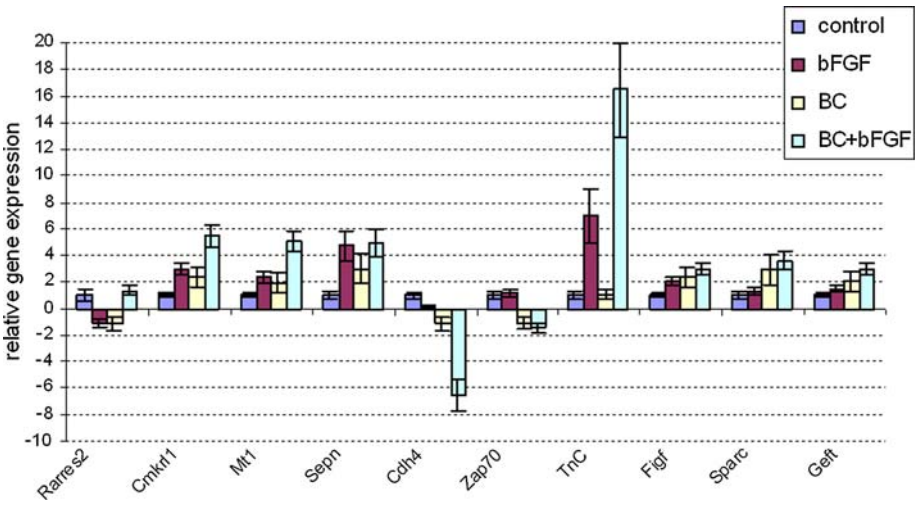



**Fig. 3** Influence of BC-containing diet on relative expression of genes related to vascular network formation in angiogenesis model in vivo (matrigel plugs). The selected genes, which included retinoic acid receptor responder (tazarotene induced) 2 (Rarres2), chemokine-like receptor 1 (Cmklr1), metallothionein1 (Mt-1), R-cadherin (Cdh4), zeta-chain [TCR]-associated protein kinase (Zap 70), Tenascin-C (TnC), c-fos induced growth factor (Figf), secreted acidic cysteine rich glycoprotein (Sparc), selenoprotein N1 (Sepn1), DNA segment Chr10, ERATO Doi 610 (D10Ertd610, Geft) were analysed by real-time PCR. Data are expressed as relative gene expression versus control. Values are shown as mean ± SD, significance $P < 0.05$ ($n = 4$)

(Sparc) and components of Ras/Rho/Rac-signalling pathway such as DNA segment Chr10 and ERATO Doi 610 (D10Ertd610, Geft) that control cell chemotaxis.

## Discussion

We have previously demonstrated that BC, at the physiological concentrations found in the human blood, activates EPCs and HUVECs chemotaxis accompanied by the induction of the genes that regulate cell adhesion and homing, but they do not alter expression of the markers of endothelial cell final differentiation [7, 9, 15].

The results presented here show that the chemotactic effect of BC occurs not only in in vitro studies but also in in vivo angiogenesis model. In in vivo studies, we observed that BC injected locally subcutaneously with matrigel augmented the angiogenic response initiated by bFGF in Balb/c mice.

In order to estimate the effect of BC in diet on angiogenesis in vivo, mice were fed with the diet that contained 12% of BC. It has been shown that rodents, in contrast to humans, can absorb and accumulate BC in tissues if their diet contains supra-physiological levels of carotenoids (≥0.02% of diet) [11]. In theory, feeding mice with a 0.02% BC diet is equivalent to a 70 kg person eating 163 carrots per day when normalized for body weight, but of course such amount is not achievable by a human being in real life. On the other hand, it is impossible to recalculate in direct way the amount of supplemented BC in mice and human diet, because the metabolism and conversion of BC differs in both species. It is well know that mice model is not recommended for the BC absorption analysis, and it was confirmed that in these animals the absorption of BC is significantly less efficient in comparison with human [11]. Therefore, in presented study 12% BC diet was supplied to mice in order to observe the effects of BC when it could not be completely metabolized. We demonstrated that BC concentration in serum increased after feeding the mice with BC-enriched diet (mean 0.69 ± 0.14 μmol/L), and no adverse effects were observed. Although the observed biological effects, in the investigated animals, could be due to (at least in part) the conversion of a proportion of BC into retinoic acid (RA), the significant increase in the BC blood level, and not RA, in the mice fed with BC-enriched diet in comparison with the control group, caused that the observations were focused only on direct BC effect. The higher amounts of BC in blood were accompanied by the activation of angiogenic response in the matrigel model. Our analysis of gene expression in matrigel plug cells isolated from the mice on high-BC diet documented BC effects on several important steps of angiogenesis such as detachment, proliferation, migration and homing of cells. The microarray data analysis pointed to the activation of genes related to cell-to-cell adhesion, cell-to-matrix adhesion, up-regulation of matrix digesting proteases, inhibition of genes that regulate cell proliferation, activation of G-protein-signalling pathway and up-regulation of genes in Ras-signalling pathway. BC diet supplementation resulted in the stimulation of GPCRs (G-protein coupled receptors), their activators and small GTPases, it also changed the expression of few genes coding for extracellular matrix

proteins (i.e. metalloproteinases). Such an alteration of gene expression profile may result in cytoskeletal rearrangements that influence cell motility, shape changes and contraction. One of the possible explanations is previously reported Rho GTPases regulation of phosphorylation of the myosin light chains (MLCs) that promote actin–myosin interaction [20].

Real-time PCR confirmed up-regulation of the gene encoding apoptosis inhibitor Mt1 and several other genes related to cell homing (Cdh4, TnC), activation of angiogenesis (Figf, TnC) and increased chemotaxis (D10Ertd610/Geft). Thus, the proangiogenic activity of BC (or BC with bFGF) in vivo seems to result from the inhibition of apoptosis and stimulation of cell chemotaxis/homing instead of activation of cell proliferation. The observed effects could be partially mediated by products of BC cleavage (retinoids) since a gene encoding Cmklr1 (RARRES2 receptor) was up-regulated in the matrigel plug cells from the mice fed with BC-enriched diet.

The activation of genes involved in chemotaxis confirms the previously found biological effect—activation of endothelial cell chemotaxis by BC [7]. Other up-regulated processes included proangiogenic extracellular matrix components, VEGF-signalling pathway, G-protein coupled receptors (GPCRs), components of the targeted Ras/Rho-signalling pathway and small GTPases, regulators of Ras/Rho pathway. The presented results confirm the previous observations on human as well as on cell cultures [3, 7, 9, 13, 18]. Clinical studies, undertaken to test the efficacy of BC supplementation for the prevention of cancer, have revealed that administration of large BC doses increase risk of lung cancer, especially in smokers [3, 13]. Further observations connected this phenomenon with the proangiogenic activity of BC in the presence of inflammation or hypoxia [18].

Together, the data presented earlier, provide a potential link to the activation of differentiated cells (HUVECs) as well as to chemotaxis and homing of progenitor cells by BC (and partially its metabolites) during angiogenesis.

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**Conflict of interest statement** There is no conflict of interest.

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