

β -Carotene and angiogenesis*

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Abstract: Carotenoids and retinoids modulate growth and differentiation of a variety of cell types and are fundamental regulators of development. Endothelial cells play an important role in angiogenesis, which is essential for organogenesis and tissue remodeling, but also inflammatory response or carcinogenesis. Binding to the retinoid (RARs) or rexinoid (RXRs) receptors, all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, and synthetic retinoids and rexinoids showed antiangiogenic properties in several models. However, the role of β -carotene in endothelial cell function and angiogenesis is still poorly characterized. Although in our experiments, β -carotene used in nontoxic concentrations (up to 3 μ M) had no detectable effect on the proliferation or apoptosis of HUVECs or umbilical-cord-blood-derived endothelial progenitors; β -carotene did not change the tubulogenic activity of cells in an in vitro angiogenesis model, but it potently activated the migration of endothelial and progenitor cells. β -Carotene also promoted the development of microcapillaries in a matrigel plug injected subcutaneously into mice. The analysis of microarray data from endothelial cells revealed that β -carotene modified the expression of genes involved in activation of chemotaxis, cell/cell and cell/matrix adhesion, matrix reorganization, G-protein-regulated intracellular signaling as well as genes involved in the rapid remodeling of the actin cytoskeleton. We conclude that physiological levels of β -carotene stimulate early steps of angiogenic activity of endothelial cells by activation of cellular migration as well as matrix reorganization and reduction of cell adhesion.

Keywords: β -carotene; angiogenesis; endothelium; microarrays; chemotaxis.

INTRODUCTION

Vasculogenesis and angiogenesis play essential roles in a number of physiologic and pathologic events, such as fetal development, vascular remodeling of growth, ischemia, inflammation, diabetic retinopathy, as well as progression of growth and invasiveness of solid tumors [1]. Carotenoids, retinol, and

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retinoids are fundamental regulators of cell growth, differentiation, and development. Retinoids and rexinoids, which bind and activate nuclear receptors RARs/RXRs, were reported to mediate the suppression of the invasive processes and reduce the incidence of metastasis in patients with head and neck, lung, or liver cancer [1]. This effect could partially be explained by the reported antiangiogenic activity. All-*trans*-retinoic acid (ATRA) was found to inhibit the vascular endothelium growth factor (VEGF), transforming growth factor (TGF- β), or IL-8-induced chemotaxis of endothelial cells without affecting cell proliferation [2]. 13-*cis* RA and 9-*cis*-RA were found to synergize with interferons in the inhibition of angiogenesis in Kaposi's sarcoma, breast, vulval, and several mouse models of the carcinoma-associated angiogenesis [3,4]. Activated RARs/RXRs interact with cognate response elements of gene promoters and regulate the gene expression. However, they may also interfere with other cellular signaling pathways. ATRA interferes not only with the CCAT/enhancer binding protein β (CEBP), but also with AP-1 and NF κ B, or Sp-1 [5,6]. It was shown that ATRA interferes with the AP-1 binding, inhibiting the expression of gene coding for proangiogenic factors, such as VEGF, stromelysin, collagenases, and TGF- β . On the other side, the activation of signal transducer and activator of transcription (STAT) pathways is suggested for the vitamin A-dependent up-regulation of antiangiogenic thrombospondin-1 and pigment epithelium derived factor (PEDF) genes in murine cells. ATRA and 9-*cis*-RA were also found to up-regulate urokinase plasminogen activator (uPA), the main proteolytic enzyme associated with the initiation of angiogenesis, in human microvascular endothelial cells.

A number of growth factors and their receptors, such as VEGF, bFGF, TGF β , platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), as well as numerous other cell/matrix (integrins), cell/cell (VE-cadherins, catenins, endoglyns, ephrins, or jagged/notch pathway) interactions, regulate the most important steps in angiogenesis, including detachment, proliferation, migration, homing, and differentiation of the vascular wall cells, mainly endothelial cells or their progenitors [7,8].

Although the antiangiogenic properties of the pharmacological levels of RA acting via RAR/RXR are well documented in several experimental models [2–7], the effect of β -carotene, or even vitamin A, on the proangiogenic activity of human endothelial cells still remains controversial.

Since 15 % of ingested β -carotene reach target cells as a nonmetabolized form in humans [9], and angiogenesis is an important part of remodeling of an ischemic tissue, as well as solid tumor malignancy [1], this study was undertaken to define the direct effects of β -carotene on endothelial cells in terms of angiogenic activity and regulation of gene expression in both in vitro and in vivo models.

METHODS

The high-performance liquid chromatography (HPLC)-grade β -carotene was kindly provided by Roche Vitamins AG, Kaiseraugust, Switzerland. The compound was stored in 1-mg aliquots in nitrogen-filled vials made of dark glass.

Cell culture

Primary endothelial cells (HUVECs) were isolated from human umbilical veins with collagenase digestion. HUVECs were cultured in EBM (EGM Bullet Kit, Clonetics) with supplements: hEGF (10 ng ml⁻¹), hydrocortisone (1 ng ml⁻¹), bovine brain extract (12 μ g ml⁻¹), antibiotics: gentamicin (50 μ g ml⁻¹) as well as amphotericin-B (50 ng ml⁻¹) and 20 % fetal bovine serum (Clonetics). The resulting cell lines were characterized by morphological and immunohistochemical criteria, such as desmin, alpha-actin, and vWF. Experiments were performed on 70 % confluent cell cultures (up to 5th passage).

The umbilical progenitor cells (UPCs) were harvested from the mononuclear cell fraction of human umbilical cord blood (within 3 h after delivery), using centrifugation gradient (Histopaque 1.077 g l⁻¹). AC133 positive cells were isolated with the Mini-MACS magnetic beads method, which allows for collection of a high-purity (about 96 %) fraction. The purity of each isolation was confirmed

by flow cytometry. The cells were subsequently cultured in EBM medium containing 10 % bovine calf serum with antibiotics. The proangiogenic conditions were achieved by culturing UPCs with VEGF (50 ng ml⁻¹) and stem cell factor (SCF: 100 ng ml⁻¹) up to 14 days at 37 °C in 95 % CO₂/5 % O₂ humid atmosphere (Jouan IG 150). Changes in the expression of cell surface antigens (CD34, AC133, KDR, VE-cadherin, CD36, CD14/45) were monitored during the culturing period by flow cytometry method. Following the stabilization of expression of the above-listed antigens (day 5 of culturing in proangiogenic conditions), the cells were defined as endothelial progenitor cells (EPCs), and used for the study.

For cell culturing, the 4 mM β -carotene stock solution in tetrahydrofuran (THF) (Sigma) was further diluted in ethanol (1:1 v/v THF/EtOH ratio) to obtain 2 mM concentration. Further dilutions were made with tissue culture medium (EBM). The final concentration of THF/EtOH in the cell culture medium was 0.075 %. HUVECs were incubated with the solvent (0.075 % THF/EtOH) containing medium (as the control) and β -carotene (0.3–10 μ M) for 24 h at 37 °C in 95 % CO₂/5 % O₂ humid atmosphere (Jouan IG 150).

β -Carotene uptake by HUVECs (measured by HPLC)

All solvents used for HPLC (THF, ethanol, *t*BME (*tert*-butyl methyl ether) (Sigma) and BHT (2,6-Di-*tert*-butyl-*p*-cresol) (Sigma) were analytical grade. THF and ethanol were additionally filtered by the aluminum oxide column (Sigma) before use. Following 24-h incubation of HUVECs with the studied compounds, cells were detached by trypsinization and washed three times with Ca⁺²- and Mg⁺²-free (phosphate-buffered saline) PBS. The cells were counted using a Burkner chamber and spinned down. The resulting pellet was frozen (-80 °C) for further analysis. β -Carotene concentration in the cell pellet and stability in the culture medium (lack of spontaneous degradation products) were assessed with the HPLC micromethod developed by Roche Vitamins AG (Kaiseraugust, Switzerland). Briefly, for extraction, 200 μ l of acetone (with 0.025 % BHT f.c.) was added to a frozen cell pellet, mixed for 1 min and dried in a vacuum centrifuge under argon (30 min at 50 mbar). 200 μ l of the β -carotene extraction solvent (ethanol/THF/*t*BME 9:1:5, 0.025 % BHT f.c.) were applied to the dried pellet, vigorously mixed, and centrifuged (3 min, 8000 \times g, 4 °C). The supernatant was used for HPLC.

For the measurement of β -carotene content in the tissue culture medium, 25 μ l of the medium was mixed with 225 μ l of the above-detailed β -carotene extraction solvent, mixed for 1 min and centrifuged for 3 min at 8000 \times g, 4 °C. The HPLC sample analysis was isocratically performed on a Vydac 218TP54 column C-18 (Roche) (4.6 \times 250 mm) at a constant column temperature 20 °C. The sample solvent consisted of ethanol/THF/*t*BME 9:1:5, 0.025 % BHT f.c. The mobile phase was: acetonitrile/*t*BME/ammonium acetate (80 mM)/triethylamine (73:20:7:0.05) at a constant flow of 1.5 ml min⁻¹. Shimadzu SCL-10AVP instrument (Shimadzu, Kyoto, Japan) with the SPD-10AV detector set at 450 nm for the carotenoid's estimation. This HPLC system allows for the detection of 12'-apocarotenal, 8'-apocarotenal, 4'- β -apocarotenal, all-*trans*- β -carotene, and (*Z*)- β -carotene. The amount of β -carotene was expressed in picomols per 10⁶ cells.

Detection of cellular toxicity and apoptosis

Possible toxic effects of compounds and solvents used in the experiments were assessed by measuring cellular levels of lactic dehydrogenase (LDH) in medium following 24-h incubation (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega).

For the estimation of apoptosis, the ApoFluor® Green Caspase Activity Assay (ICN Biomedicals, Inc.) was used. This test allows for the measurement of global cellular caspase activity. Proapoptotic staurosporine (1 nM) was used as the positive control for apoptosis. Following incubation with the fluorescent dye (1 h), cells were harvested, washed to remove unbound dye with the Ca⁺²- and Mg⁺²-free PBS and 1 \times 10⁶ cells were placed in black microtiter plates (NUNC A/S, Roskilde). The fluorescence was detected using plate reader (Synergy HT, BIO-TEK) and quantitated at 520-nm emission wave-

length following the excitation with the 490-nm light. Results were expressed as the percentage of activity detected in the control (incubated with the solvent only) HUVECs and EPCs.

HUVEC and EPC proliferation assay (the bromo-deoxyuridine (BrdU) incorporation)

In order to determine the effects of β -carotene on HUVEC and EPC proliferation, the rate of DNA synthesis was measured by incorporation of the thymidine analog, bromo-deoxyuridine (BrdU) to DNA. HUVECs or EPCs (5×10^4 cells) were incubated with β -carotene for 24 h, as described above, and additionally with BrdU for the last 3 h. Following incubation, cells were fixed and stained with the anti-BrdU kit (Roche) according to the manufacturer's recommendations. Proliferation induced by VEGF (0.2 nM) (Sigma) or bFGF (0.5 nM) (Sigma) for 24 h, served as a positive control. The results were presented as the ratio of the control BrdU incorporation to HUVECs incubated in the medium with solvents only.

Migration of cells assay (Boyden's chamber)

HUVECs or EPCs were suspended (10^5 cells/100 μ l) in the EBM medium with 0.5 % FBS and seeded into BD Falcon™ FluoroBlok™ inserts (3 μ m pore, Becton Dickinson). The inserts containing cells were placed into a 24-well plate with 600 μ l of the EBM medium and 0.5 % FBS, and incubated for 24 h at 37 °C against β -carotene (3 μ M), or 1-phospho-sphingosine (S1P, 500 nM as the positive control) (Sigma) [18]. After 24 h, HUVECs were stained with anti-CD31 antibody conjugated with phycoerythrin (PE) (10 μ g ml⁻¹) (Becton Dickinson) for 30 min at 37 °C. Subsequently, cells in the inserts were washed with the Ca⁺²- and Mg⁺²-free PBS, and the fluorescence of cells, which had migrated through the pores to the bottom side of the inserts, was measured with a fluorescence plate reader (Synergy HT, BIO-TEK). The changes in intensity of fluorescence in a sample with agents vs. control (cells which migrated against the EBM with solvents only) corresponded to the amount of migrating cells. For the assessment of migrating EPCs, flow-cytometry was used. The chemotactic activity of the cells was expressed as the chemotaxis index (CHI), which represents the ratio of migration stimulated by the studied compound vs. random, unstimulated migration of cells in the solvent (THF/EtOH) control sample.

3D in vitro model of tubulogenesis in matrigel [10]

HUVECs or EPCs were resuspended in matrigel (containing laminin, collagen IV, entactin, heparan sulfate proteoglycans) (Becton Dickinson) to the final concentration of 1×10^6 cells ml⁻¹. The cell-matrigel mixture (50 μ l) was placed in the cell culture dishes and incubated in a humidified CO₂ incubator (Jouan) at 37 °C for 30 min. Subsequently, the EBM medium (100 μ l) supplemented with solvent (control probe), or β -carotene (3 μ M) was applied on top of the matrigel with immersed cells and incubated in a humidified CO₂ incubator for 24 h at 37 °C. The number of tubules formed in the presence of VEGF (0.2 nM) or bFGF (0.5 nM) served as the positive control. Formation of tubules by cells suspended in matrigel was assessed under the light microscope (at magnitude $\times 10$) and photographed. Lengths of the analyzed tubule-like structures were calculated and expressed as average sum of total length of tubules visible under the light microscope.

Angiogenesis in vivo (the mouse model)

Protocol was accepted by DSM Company (Basel, Switzerland, Dr. R. Goralczyk) and the local University Ethics Committee. All animal experiments were performed according to Polish law and approved by the Polish Animal Inspectorate and Institutional Animal Care. Diet production was performed at DSM Nutritional Products. Female Balb/c mice ($n = 10$ for group) were fed for 5 weeks with

two different diet schedules (with or without β -carotene supplementation). *β-Carotene-supplemented diet*: Kliba 2415 (vitamin A 1400 IU kg⁻¹ β -carotene Beadlet 10 % = 1200 ppm β -carotene). *β-Carotene-non-supplemented diet*: Kliba 2415 (vitamin A 1400 IU kg⁻¹ Placebo Beadlets = 0 ppm β -Carotene). Female Balb/c mice ($n = 6$) received subcutaneously sterile injections of $2 \times 500 \mu\text{l}$ matrigel (Becton Dickinson) (dorsally). Parallel matrigel plug contained bFGF (50 nM). Six days later, animals were sacrificed by overdosing urethane anesthesia and the matrigel plugs were removed, fixed, and immersed in paraffin. Immunohistochemistry was performed using routine protocol. Primary anti-CD31 antibody (anti-PECAM-1, BD Pharmingen) at 1:300 dilution were used. The slides were rehydrated and incubated in 3 % peroxide solution for 10 min to block endogenous peroxidase activity. The Streptavidin-Biotin (BD Pharmingen) detection system was used. DAB was used as chromogen. The slides were contra-stained with Mayer hematoxylin (DAKO, Denmark). The amount of capillaries was counted under microscope in five different fields in each of the three slices taken from different parts of each plugs. The number of capillaries detected in slices from the plugs was counted by the specialist-morphologist in the blind manner and expressed as the number of vessels with or without the lumen. The number of separate endothelial PECAM-positive single cells not connected to the capillaries, migrating to matrigel was counted separately.

Protein synthesis (Western blot)

Western blot was used to detect the synthesis of interleukin 8 (IL-8) by HUVECs incubated with the studied compounds.

The cells were rinsed with the Ca⁺²- and Mg⁺²-free PBS and lysed in CelLyticTM-M Mammalian Cell Lysis/Extraction Reagent (Sigma) containing protease inhibitors (Protease Inhibitor Cocktail Tablets completeTM, Roche). Protein content was determined by the Bradford method. Proteins were electrophoretically separated on a SDS-polyacrylamide gel under reducing conditions and transferred to PVDF (polyvinylidene (di)fluoride) membranes (BIORAD). Transfer was performed in 100 V for 2 h. Blots, stained with 2 % Ponceau S (Sigma), in 3 % trichloroacetic acid to visualize proteins, were saturated with 1 % blocking solution (Lumi-Light^{PLUS} Western Blotting Kit Mouse/Rabbit, Amersham Pharmacia Biotech) and incubated for 1 h with mouse anti-IL-8 antibody (Mouse monoclonal anti-IL-8 antibody [B-2]). Following washing in TBST (Lumi-Light^{PLUS} Western Blotting Kit Mouse/Rabbit), membranes were subsequently incubated for 30 min with anti-mouse Ig-POD Fab fragments (Lumi-Light^{PLUS} Western Blotting Kit Mouse/Rabbit, Roche) or monoclonal anti-goat/sheep IgG Monoclonal Anti-Goat/Sheep IgG Clone GT-34 peroxidase conjugated (Sigma). Monoclonal antibodies against actin were used as a control of protein loading (Mouse monoclonal anti-actin antibodies—Actin [C-2], Santa Cruz Biotechnology, Inc.).

Enhanced chemiluminescence, performed according to the manufacturer's instructions (Amersham) was used to demonstrate positive bands, which were visualized after exposure on a transparent medical X-ray film.

Isolation of total RNA

Following 24-h incubation with the studied compounds, total RNA was isolated from cells by the guanidine thiocyanate-caesium chloride method using Trizol (Invitrogen Life Technologies) and purified with the SV total RNA Isolation System Kit (Promega). The quality of RNA was confirmed by denaturing gel electrophoresis and analysis on the Agilent 2100 Bioanalyser (Agilent Technologies).

Microarray Affymetrix HG-U133A hybridization

The aim of the microarray experiments was to screen gene expression pattern changes in response to β -carotene in HUVECs and EPCs. For the microarray hybridization, RNA was reverse transcribed into

cDNA with a primer containing the T7 promoter, using Superscript II (Invitrogen Life Technologies). cDNA was used as a template in a biotin-labeled transcription reaction (Enzo BioArray, Affymetrix). The resulting target cRNA was purified on RNeasy columns (QIAGEN) and fragmented for hybridization to Affymetrix HG-U133A GeneChips. Hybridization was done overnight at 45 °C for 16 h in the GeneChip Hybridization Oven 640 (Affymetrix). The GeneChips were subsequently processed on the Affymetrix GeneChip Fluidics Workstation 400 according to the EukGE-WS2v4 protocol. The GeneChips were scanned with the Hewlett Packard GeneArray Scanner, and the results analyzed using Affymetrix Microarray Analysis Suit 4.0.

Analysis of the microarray data

Changes in relative gene expression were calculated vs. control (THF/EtOH solvent). Only spots with significant differences in signal intensity (more than 1.4-fold and only when the p-value was at least 0.05) were included in the analysis.

In order to identify genes, which belong to pathways regulated by β -carotene, the search of promoter sequences of the identified genes was performed to detect similarities in transcription factors binding sites. The 4-kb upstream sequences relative to the transcription start site were retrieved from Database of Transcriptional Start Sites DBTSS (<http://dbtss.hgc.jp/index.html>). Searches of transcription factor binding sites were done using the TRANSFAC database at a default threshold setting of 90 (<<http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>>).

cDNA synthesis and quantitative real-time PCR (qRT-PCR)

In order to verify and validate gene expression changes identified in the microarray experiments, quantitative expression analysis of IL-8, CXCR4, VCAM-1, EGR-1, MAD1L1, BIRC5, and MEOX2 was performed by qRT-PCR using GAPDH as the reference gene. For the cDNA synthesis, 1 μ g of total RNA was reverse transcribed at 42 °C for 50 min in a total volume of 40 μ l reaction buffer containing 5 x First Strand Buffer, DTT, oligo(dT) (Sigma), deoxy-NTPs (Promega), and 200 units of SUPERSCRIPT II reverse transcriptase (Invitrogen Life Technologies). The reaction mixture was heated to 70 °C for 15 min and immediately chilled on ice. Subsequently, cDNA was subjected to real-time PCR in a reaction mixture containing QuantiTect SYBR Green PCR (Qiagen) mix and primers. The sequences of the primers used in this study are presented in Table 1. The primers were designed to include an intervening intron between the sense and antisense primers, thereby eliminating the possibility of amplifying any genomic DNA, and checked for specificity by BLAST searches. All real-time PCR reactions were performed on the DNA Engine Opticon II (MJ Research). The thermal profile 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⁻¹ from 35 to 95 °C. A negative control without cDNA template was run with every assay to ensure overall specificity. The expression rates were calculated as the normalized CT difference between a control probe and sample with the adjustment for the amplification efficiency relative to the expression level of the housekeeping gene GAPDH. Calculation was performed using the Calculation Matrix software for PCR Efficiency REST-XL (gene.quantification@wzw.turn.de).

Table 1 List of primes used for the study (qRT-PCR).

A. Endothelial progenitor cells (EPCs)			
Gene symbol	mRNA sequence	Forward primer	Reverse primer
MAD1L1	NM_003550	5'-ACAGTCTCTGTAATCGCGAAAGC-3'	5'-TCAGGGTGGATAAAAACCATGGT-3'
EGR1	NM_001964	5'-AGCACCTGACCGCAGAGTCT-3'	5'-GGTCTCCACCAGCACCTTCTC-3'
BIRC3	NM_182962	5'-TGCCAAGTGGTTCCAAGGT-3'	5'-GGCTTGAACCTGACGGATGAA-3'
IL-8	NM_000584	5'-CTGGCCGTGGCTCTCTGG-3'	5'-TCCTGCGTGTAGTTATCTGAAGTGTATATAC-3'
CXCL12	NM_199168	5'-AGCCAACGTCAAGCATCTCAA-3'	5'-TCCTCAGTCGGGCTACAATC5'
CXCR4	NM_003467	5'-CACCGCATCTGGAGAACCA-3'	5'-TCCTGCGTGTAGTTATCTGAAGTGTATATAC-3'
CCL2	NM_002982	5'-CTCGCTCAGCCAGATGCAA-3'	5'-GCACTGAGATCTTCTATTGGTGTATGAA-3'
Jagged-1	NM_000214	5'-AAGGAGTAGATGTCACCAAGTCTT-3'	5'-GCGGAAACATCTTCAAAAATATCA-3'
Notch-4	NM_004557	5'-CACTAGGCGAGGACAGCATTG-3'	5'-CAACTCCATCCTCATCAACTTCTG-3'
CD36	NM_000072	5'-ACAGATGCAGCCTCATTTCC-3'	5'-CGTCGGATCAAATACAGCA-3'
TSP1	NM_003246	5'-CGGATCAGCTGGACTCTGA-3'	5'-TGTCTGGTGGCCATCTTC-3'
KDR	NM_002253	5'-TCCCCTTAGAAGAACCAGAA-3'	5'-CTGAGGCAAGAACCATACCA3'
vWF	NM_000552	5'-GATGGAGTCCAGCACCAGTT-3'	5'-GTCACTTCACACAGGCCACA-3'
eNOS	NM_000603	5'-CATCACCAGGAAGAAGACCTTTAAA-3'	5'-TCACTCGCTTCGCCATCA-3'
BAX	NM_004324	5'-GAAGCTGAGCGAGTGTCTCAAG-3'	5'-GTCCACGGCGGCAATC-3'
GAPDH	NM_002046	5'-GCCAGCCGAGCCACATC-3'	5'-GCGCCAATACGACCAAAA-3'
B. Human umbilical vein endothelial cells (HUVECs)			
Gene symbol	mRNA sequence	Forward primer	Reverse primer
MEOX2	NM_005924	5'-CTGCGGAGGCGGAGAA-3'	5'-GTAATTTCTTCTCTGGGAGTCTGA-3'
MAD1L1	NM_003550	5'-ACAGTCTCTGTAATCGCGAAAGC-3'	5'-TCAGGGTGGATAAAAACCATGGT-3'
EGR1	NM_001964	5'-AGCACCTGACCGCAGAGTCT-3'	5'-GGTCTCCACCAGCACCTTCTC-3'
CXCR4	NM_003467	5'-CACCGCATCTGGAGAACCA-3'	5'-TCCTGCGTGTAGTTATCTGAAGTGTATATAC-3'
IL-8	NM_000584	5'-CTGGCCGTGGCTCTCTGG-3'	5'-TCCTGCGTGTAGTTATCTGAAGTGTATATAC-3'
VCAM1	NM_080682	5'-AGGCTGGAAGAAGAAGCGGAAAGG-3'	5'-AGCTGTAAGTTTTATGTCTTTTGGAGTAAC-3'
BIRC5	NM_001168	5'-TTAACCTTGGTGAATTTTGAAGACT-3'	5'-TTCTTATTGTTGGTTTCTTTTGCAA-3'
BCDO	NM_017429	5'-TGCCAGCCGGAATTTCTTTAT-3'	5'-TCGGTATTGCTTTCCATTGTGA-3'
GAPDH	NM_002046	5'-GCCAGCCGAGCCACATC-3'	5'-GCGCCAATACGACCAAAA-3'

Statistical analysis

Statistical analysis was performed with Microsoft EXCEL 5 software and by one-way ANOVA. All results are expressed as mean values \pm standard error of the mean (SEM). All statistical analyses were preceded by tests for normal distribution and homogeneity of variables. Parameters that did not fulfill these tests were logarithmically transformed. Statistical comparisons were made by unpaired t-tests for comparisons of quantitative variables. $P < 0.05$ was considered significant.

Bioinformatics

Sequence data were assembled and analyzed using promotor sequences retrieved from the Database of Transcriptional Start Sites DBTSS (<http://dbtss.hgc.jp/index.html>) web site transcription factor consensus sequences and databases were accessed using the TRANSFAC (<http://transfac.gbf.de/TRANSFAC/>) web site. Calculation of real-time PCR reaction efficiency was performed using the program REST-XL (gene.quantification@wzw.turn.de)

RESULTS

Differentiation of the early EPCs

The 14-day incubation, in suspension, of umbilical cord blood AC133 positive cells under proangiogenic conditions (VEGF 50 ng ml⁻¹, SCF 100 ng ml⁻¹) resulted in the changes in expression of surface antigens characteristic for endothelial progenitor (EPC)-like cells (Fig. 1). The expression of the VEGF receptor KDR remained unchanged, when the expression of VE cadherin (characteristic for the endothelium) [11] was significantly up-regulated. The amount of CD34 and AC133 antigens decreased gradually in accordance with the disappearance of the marker of myeloid cells CD14/45 (Fig. 1). The significant changes in the antigen expression were seen as soon as 48 h of incubation and stabilized on day 5 (Fig. 1). Following 5-day incubation with VEGF and SCF, the non-adhering 2 × 10⁶ ml⁻¹ cells were used for further experiments.

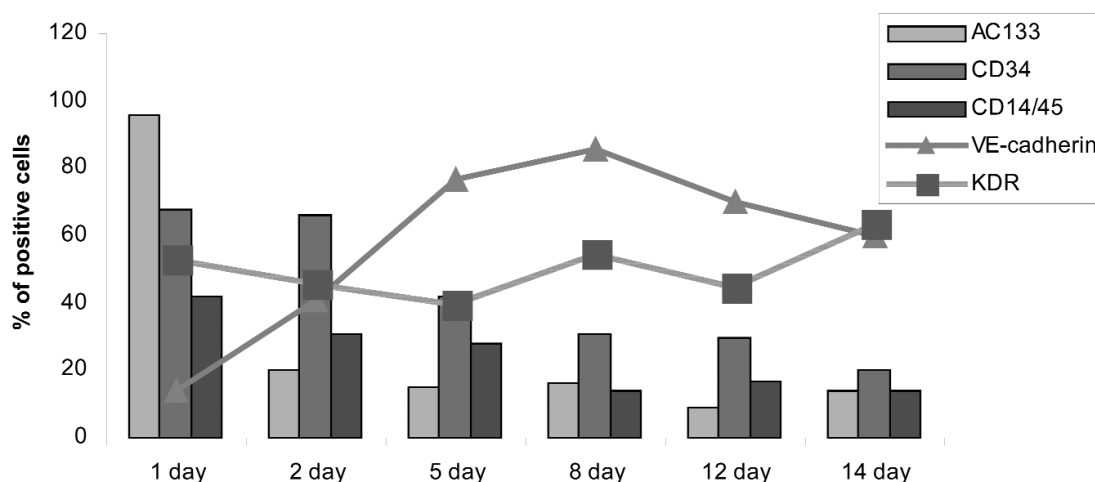


Fig. 1 The percentage of the AC 133 positive umbilical cord blood harvested cells, that expressed the surface receptors characteristic for endothelial cells (VE-cadherin, KDR), and for progenitors (CD34, CD14, CD14/45, AC 133) surface antigens after the 14-day incubation with proangiogenic medium (VEGF 50 ng ml⁻¹, SCF 100 ng ml⁻¹). Mean ±SD from 3–5 experiments. **p* at least < 0.05 vs. 1st day.

Uptake of β-carotene by HUVECs and EPCs

β-Carotene uptake by cells in culture was concentration- and time-dependent (data not shown). β-Carotene concentration of 3 μM was used for further experiments, since the higher concentrations of β-carotene caused endothelial cell toxicity as evidenced by the LDH leakage from cells (especially EPCs) after 24-h incubation (data not shown).

Effects of β-carotene on cell apoptosis

Unlike cells treated with proapoptotic staurosporine (1 nM), no proapoptotic activity measured by caspase activation was detected in HUVECs or EPCs incubated for 24 h with β-carotene (up to 10 μM), as well as with THF/EtOH used as the solvent. However, β-carotene in concentration higher than 10 μM induced cell apoptosis, especially in EPCs (data not shown).

Effect of β-carotene on cell proliferation

Unlike VEGF (0.2 nM) and bFGF (0.5 nM), which stimulated cell proliferation, β-carotene used in the nontoxic concentration (below 10 μM) did not influence HUVEC or EPC proliferation as measured by the BrdU incorporation (Table 2).

Table 2 The lack of influence of β-carotene on cell proliferation (results expressed as the percent of the control BrdU incorporation (mean ± SD from 5–10 experiments; **p* < 0.01 at least vs. control)).

	Control (THF/EtOH)	VEGF (0.2 nM)	BFGF (0.5 nM)	BC (3 μM)
EPC	100 ± 15	226 ± 16	242 ± 15	98 ± 17
HUVEC	100 ± 10	216 ± 18	246 ± 22	91 ± 15

Effect of β-carotene on cell migration

β-Carotene (3 μM) led to a four-fold increase in HUVEC chemotaxis (Fig. 2); this response was even higher (six-fold) in EPCs (Fig. 2B). Sphingosine-1-phosphate (S1P), a potent activator of endothelial cell migration, was used as the positive control to confirm the chemotactic cell potential in our assay.

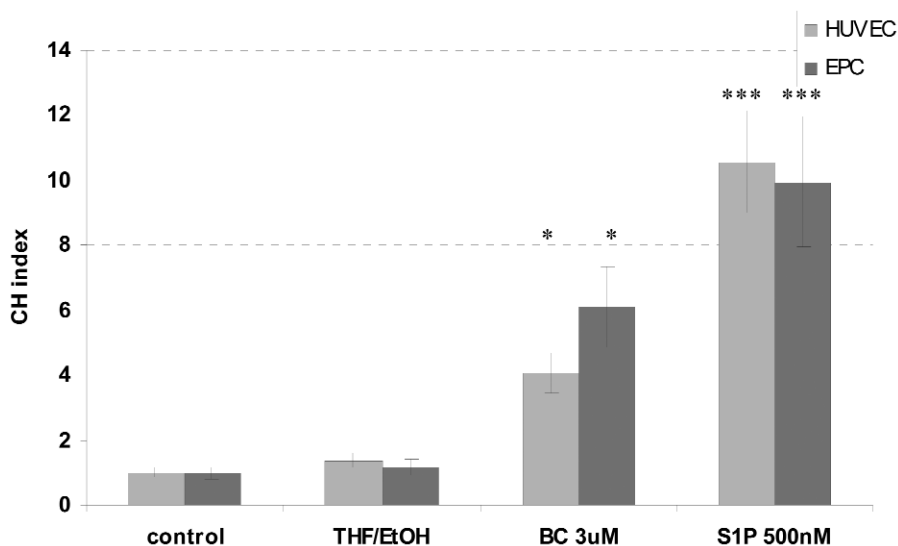


Fig. 2 The β-carotene-induced chemotaxis of HUVECs and EPCs. Cells were seeded onto upper well membranes of Transwell plate inserts and then placed into wells prefilled with EBM medium with 0.5 % FBS containing the chemoattractant β-carotene 3 μM or sphingosine-1-phosphate (S1P) 500 nM as the positive control for chemotactic activity. After incubation for 24 h, cells that had migrated across micropore inserts (5-μm pore size) to the lower surface of the membranes were stained by anti-human CD31 antibody coupled with phycoerythrin (PE) for HUVEC and by flow-cytometry for EPC. Chemotaxis index: the ratio of stimulated migration divided by that of basal, unstimulated migration of cells in control medium sample. Values are mean values ±SD, *n* = 3 done in triplicates. *Significantly different from the corresponding control, **P* < 0.05, ***P* < 0.005

Effect of β -carotene on tubulogenesis in the 3D matrigel in vitro model

The 3D matrigel assay of tubulogenesis [11] was used to verify the angiogenic property of β -carotene in vitro. Only trace tubulogenic activity was detected in the cells cultured in matrigel covered with medium without factors. VEGF (0.2 nM) and bFGF (0.5 nM) potently increased the number of tubules in matrigel with HUVECs, however not in the EPC suspension. No tubulogenic activity of β -carotene was observed in this in vitro model (Fig. 3).

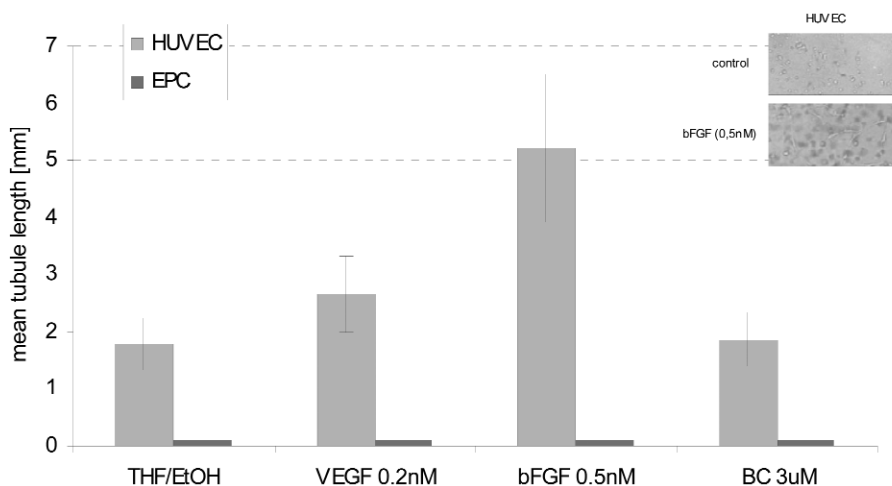
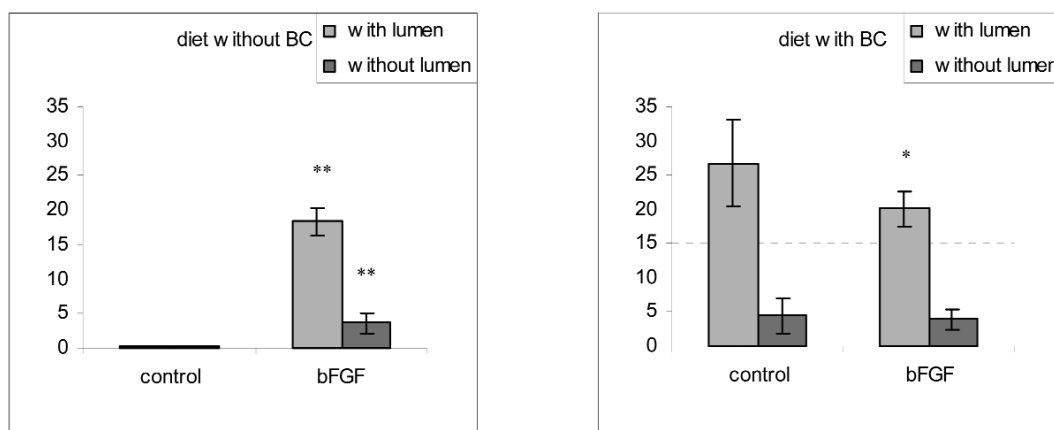


Fig. 3 The lack of influence of β -carotene on tube-like structure formation in vitro in the 3D matrigel model by HUVECs as well as by EPCs. Formation of microtubules by HUVECs or EPCs suspended in matrigel after incubation with β -carotene (3 μ M) or (as positive controls) VEGF (0.2 nM), or bFGF (0.5 nM) for 24 h was investigated. Tube-like structures were observed under the optical microscope (at magnitude $\times 10$) and photographed. The sum of length of the analyzed tubule-like structures was calculated and expressed as average sum of total length of tubules visible under the optical microscope in the random five fields of each matrigel. Values are mean values \pm SD, $n = 10$ done in triplicates. Significantly different from the corresponding (medium with 0.075 % THF/EtOH) control, for VEGF: $P < 0.05$, and bFGF: $P < 0.005$.

Proangiogenic activity of β -carotene in the in vivo mouse model

Six-week feeding of mice with β -carotene significantly increased the density of a capillary network (with and without a lumen) in the subcutaneously injected matrigel pad. β -Carotene alone was a potent activator of angiogenesis in vivo, and the presence of bFGF did not augment the effect of β -carotene (Fig. 4).



Diet	without BC			with BC			without vs with BC	
	control	bFGF	p-value vs control	control	bFGF	p-value vs control	p-value control vs control	p-value bFGF vs bFGF
with lumen	0.0	18.36 ± 2.08	0.001	26.75 ± 6.40	20.08 ± 2.60	0.01	0.001	0.01
without lumen	0.0	3.67 ± 1.45	0.001	4.48 ± 2.56	3.97 ± 1.52	0.08	0.01	0.4

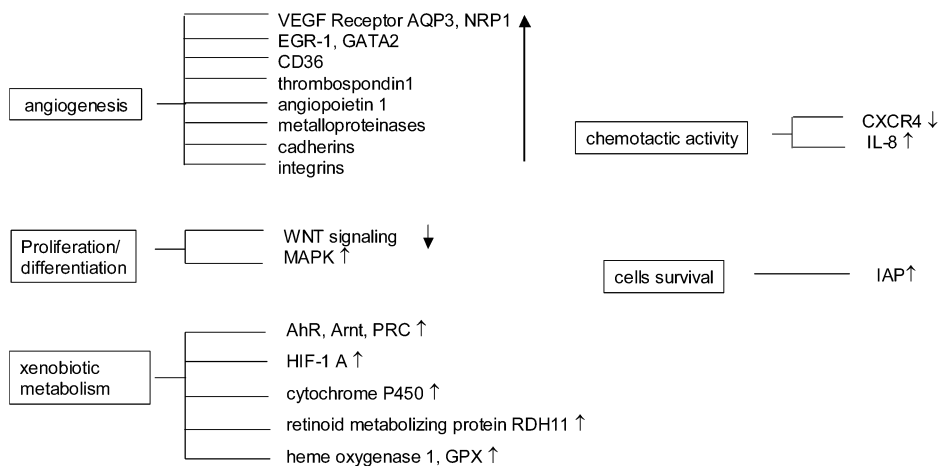
Fig. 4 The effects of β -carotene treatment on angiogenesis in mouse matrigel model in vivo. Angiogenesis was assayed in subcutaneously injected matrigel plugs (containing or not bFGF) for six days. Angiogenesis was quantified by counting the number of vessels without lumen and the number of vessels with lumen penetrating the matrigel plug, is an average of five different fields in each of the three slices taken from different regions of the each of matrigel plug. For immunostaining, paraffin sections were incubated with mouse monoclonal anti-CD31 antibody. Values are mean values \pm SD, $n = 6$ per group. Significantly different from the corresponding control is presented in the table.

Microarray (Affymetrix HG-U133A) analysis

Using the criteria described in the Methods section, we identified 838 genes in HUVECs, whose expression changed by incubation with β -carotene (446 down-regulated, 393 up-regulated). In EPCs, β -carotene changed the expression of 1816 genes, most of them (1140) were up-regulated, while 676 were down-regulated. The analysis of the microarray data, including genes regulated (up or down) from at least 1.4- up to 10-fold, provided a list of significantly regulated gene coding for proteins, which belong to several cellular pathways contributing to proangiogenic activity (cell cycle, adhesion, matrix remodeling, chemotaxis), apoptosis, receptor-mediated signal transduction, as well as transcription factors and regulators of protein synthesis (zinc finger proteins, ribosomal proteins), xenobiotic metabolism, and inflammatory response (Figs. 5A and 5B).

Since endothelial cells and their progenitors show proangiogenic activity [1], special attention was paid to β -carotene-regulated genes involved in the cell cycle/proliferation, apoptosis, chemotaxis, and homing. β -Carotene weakly up-regulated the key gene coding for proteins participating in the regulation of a cell cycle, such as MCM5, MAD1L1 connected with G1/S check point and polo-like kinase Plk1, and NUCKS related to G2/M check point. β -Carotene also up-regulated important inhibitors of a cell cycle, including Wee1 and PKMyt1.

A EPC



B HUVEC

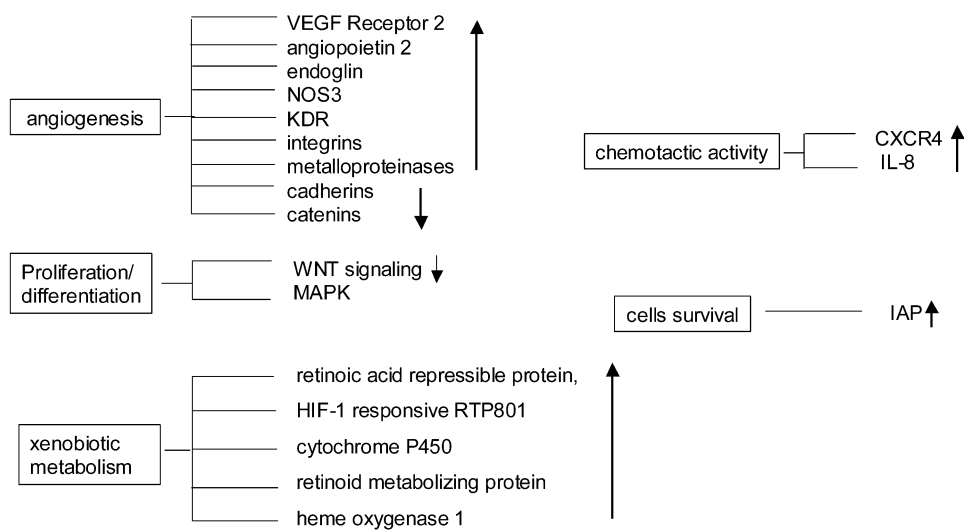


Fig. 5 The schematic presentation of microarray results. Clustering of mostly regulated by β -carotene genes related to angiogenesis in EPCs and HUVECs.

Similarly weakly, β -carotene up-regulated inhibitors of apoptosis associated with the FASL pathway, such as CFLAR (Flip), BIRC5 (IAP), and TRAF4, and down-regulated the expression of apoptosis activators OPTN, PAR1, and PAR4 [13]. The parallel up-regulation of a number of pro- and anti-apoptotic members of the Bcl-2 family was also observed. (β -Carotene up-regulated the pro-apoptotic Bcl2L11/BAM, anti-apoptotic MCL-1 and TEGT-BAX inhibitor gene expression).

The group of β -carotene-regulated genes, which code for proteins participating in cell/cell interactions, such as cadherins (CELSR1), catenins (CTNNA1L, CTNNB1), the leukocyte-endothelium adhesion mediating molecules (VCAM1, SELP, CD24) were generally down-regulated by β -carotene. On the contrary, the expression of gene coding for proteins associated with cell-extracellular matrix adhesion, such as integrins (ITGA6), and scavenger receptor SCARB1 were up-regulated.

The expression of gene encoding extracellular matrix-degrading enzymes and stimulators of chemotaxis, which may regulate the matrix-degradation, receptor shading and cell migration, such as ADAMTS1, ADAMTS18, MMP10, MMP12, MMP14, and MMP24 were differentially regulated. Down-regulation of different types of extracellular matrix components, including collagens, fibrillin 1 (FBN1), laminin β 1 (LAMB1), matrilin 2 (MATN2), and matrix Gla protein (MGP) was also observed.

The regulation of expression of gene coding for proteins involved in endothelial homing/chemotaxis (IL-8, CXCR-4) was found, too.

Among the proteins participating in intracellular signaling pathways the most evident changes in gene expression were associated with the members of the family G-protein coupled receptors (GPCR), including GPR12, CXCR4, ITGA6, DTR, the Rho-like small GTPase family or their regulators, and secreted factors such as IL-8 and CXCL2.

Following the identification of differentially regulated genes, a detailed promoter analysis of the significantly regulated genes was performed using a database of transcriptional start sites in order to recognize a common transcriptional regulatory network for the reconstitution of up/downstream signalling pathways, possibly influenced by β -carotene in HUVECs. The analysis involved the comparison of promoter sequences retrieved from the Database of Transcriptional Start Sites DBTSS (<<http://dbtss.hgc.jp/index.html><) web site transcription factor consensus sequences using the TRANSFAC database web site (<<http://transfac.gbf.de/TRANSFAC/>>). The promoter analysis revealed that β -carotene activation was related to transcription factors regulated by the p38 MAP kinase pathway (e.g., STAT, Max, cMyc, Elk1, CHOP, MEF2, ATF2, PPAR, CREB, SP-1, cJUN, cFOS, C/EBPa, and GATA) [44]. Early growth response factor (Egr-1), which was found to mediate expression of EC genes after vascular injury [45], was the most frequently recognized common transcription factor for β -carotene-regulated genes or for the transcription factors coactivator or target.

Quantitative changes in gene expression measured by qRT-PCR

In order to verify and validate the results of microarray experiments in relation to biological effects of β -carotene, the expression of selected gene was measured with qRT-PCR.

Similar direction of β -carotene regulation (up or down) of genes, whose activity is related to proliferation (MAD1L1, MEOX2) [14], chemotaxis (IL-8, CXCR-4, VCAM1) [15,16], and inhibition of tubulogenesis (MEOX2) was confirmed (Table 3). Additionally, the β -carotene-induced difference in the EGR-1 gene expression was replicated, since the up-regulation of DR1 and NAB1, inhibitors of the transcription factor EGF-1, an important factor of proangiogenic activity of endothelial cell [17], was observed (Table 3), consistent with the results of the microarray experiments.

Table 3 The influence of β -carotene (BC) on the selected from the microarray gene expression measured by qRT-PCR. EPCs: human umbilical cord-blood-derived endothelial progenitors. HUVECs: human umbilical cord vein endothelial cells ($n = 3-5$ experiments).

Gene name	EPC		HUVEC	
	BC (3 μ M)	p-value	BC (3 μ M)	p-value
BCMO	–	–	1.2 \pm 0.56	0.01
IL8	5.1 \pm 0.79	0.001	2.86 \pm 0.79	0.015
CCL2	6.54 \pm 0.84	0.001	–	–
CXCL12	2.4 \pm 0.85	0.01	–	–
CXCR4	–1.06 \pm 0.11	0.01	2.36 \pm 0.81	0.01
VCAM1	–	–	–2.51 \pm 0.84	0.034
MAD1L1	6.42 \pm 0.67	0.001	2.28 \pm 0.77	0.008
MEOX2	–	–	1.75 \pm 0.85	0.03
EGR1	2.94 \pm 0.79	0.01	2.65 \pm 0.79	0.01
BIRC3	2.45 \pm 0.77	0.013	–	–
BIRC5	–	–	1.3 \pm 0.77	0.05
CD36	4.8 \pm 0.65	0.05	–	–
TSP	2.7 \pm 0.56	0.04	–	–
KDR	2.4 \pm 0.69	0.03	–	–
vWF	1.2 \pm 0.77	0.03	–	–
eNOS	2.3 \pm 0.86	0.24	–	–
HO-1	2.1 \pm 0.56	0.02	–	–
BAX	–3.1 \pm 0.84	0.05	–	–

Relatively low basal expression of the β , β -carotene 15,15'-monooxygenase (BCMO, EC 1.13.11.21) in HUVEC was not influenced by β -carotene (measured by qRT-PCR as well as by the microarray assay), suggesting the lack of β -carotene-induced changes of β -carotene catabolism in endothelium (Table 3).

Increase of IL-8 protein release by β -carotene (Western blotting analysis)

The activation of the expression of IL-8, which possesses chemotactic properties for endothelial cells, by β -carotene was confirmed by Western blot. Densitometric analysis revealed that incubation of HUVECs with β -carotene increased the cellular IL-8 protein content after 24 h (Fig. 6).

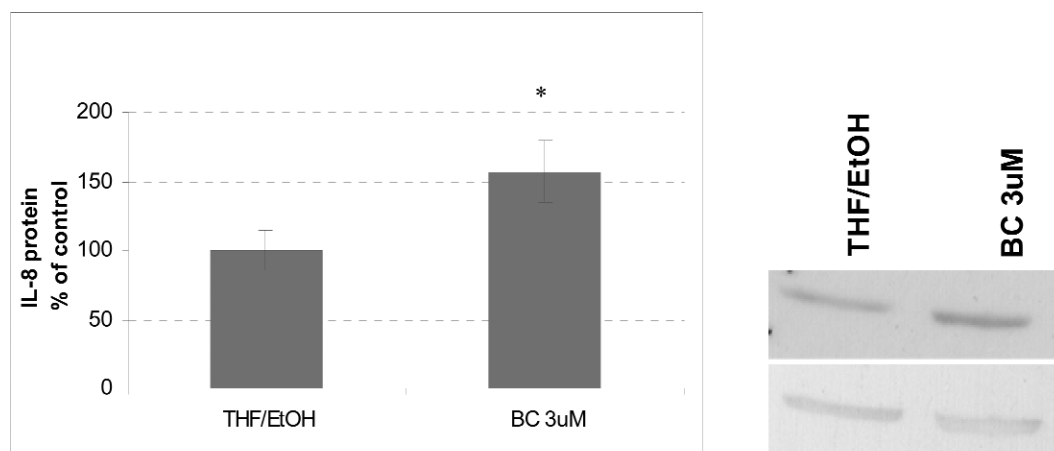


Fig. 6 Influence of β -carotene on IL-8 protein expression. Western blot analysis of protein content in HUVECs treated 24 h with EBM medium with 0.075 % THF/EtOH (control), or β -carotene 3 μ M. β -actin was used as a control of the equally loading of proteins. Representative Western blot of IL-8 protein expression. Values (quantitative evaluation, densitometric analysis) of IL-8, expressed as percent of control are mean \pm SD, $n = 6$ done in triplicates. * $P < 0.05$.

DISCUSSION

The main outcome of this study is the evidence of β -carotene-induced expression of genes involved in prochemotactic, and, thus, proangiogenic processes in endothelial cells and their progenitors.

Retinoids are a group of natural and synthetic analogs of vitamin A that can inhibit proliferation, promote differentiation, trigger apoptosis, and affect several intracellular signaling pathways through the interactions with the RAR/RXR receptor families [3,7,18,19]. The recent studies indicated that retinoid treatment is associated with inhibition of angiogenesis in in vitro and in vivo models [2–6]. The synthetic retinoid *N*-(4-hydroxy-phenyl) retinide: fenretinide (HPR) was shown to decrease tumor vessel density, repressing the release of proangiogenic VEGF, and matrix metalloproteinase MMP-2 by the tumor cells, as well as reducing endothelial cell proliferation and VEGFR-2 expression. However, unlike 13-*cis*-RA, fenretinide exerts its activity by both RAR-dependent and RAR-independent pathways. Fenretinide activity results from the activation of RAR γ and RAR β , but not RAR α . It can also be caused by the activation of acidic sphingomyelinase (ASM), and promotion of ceramide synthesis, which, metabolized to ganglioside GD3, induces 12-lipoxygenase (12-LOX)-dependent oxidative stress.

Gaetano [20] demonstrated that natural retinoids, all-*trans*-RA and 9-*cis*-RA, but not 13-*cis*-RA induced bFGF production by bovine aortic endothelial cells (BAECs), and exhibited a dose-dependent proangiogenic effect by enhancing BAEC proliferation and differentiation into tubular structures in matrigel. This effect was exclusively related to the activation of RAR α , since the treatment with the selective RAR α antagonist, RO 41-5253, reversed this effect. They also demonstrated the proangiogenic activity of ATRA in the mouse subcutaneous matrigel plug as well as in the chicken chorioallantoic models [20]. Moreover, proangiogenic activity of retinoids was reported by several other groups [8,9,18,19].

Thus, the conflicting results regarding the net effect of carotenoids and retinoids on angiogenesis may be explained by different, tissue-specific expression patterns of the RAR/RXR subfamilies, as well as by the non-receptor-related influence on the cellular signaling pathways.

β -Carotene, α -carotene, β -cryptoxanthin lutein, and lycopene belong to the well-characterized family of carotenoids, constituting approximately 90 % of the total plasma carotenoids in humans in

concentrations ranging from not detectable to 3–10 $\mu\text{mol l}^{-1}$ (dependent upon diet and/or supplementation). In our study, the uptake of β -carotene by HUVECs and EPCs was documented by HPLC. This was not related to the increase of the basal expression of β , β -carotene 15,15'-monooxygenase (BCMO), the key enzyme that cleaves β -carotene centrally into two molecules of retinal, the source of retinoic acid or retinol (vitamin A), as evidence by the results of the microarray experiments, and later confirmed by qRT-PCR. Thus, the observed effects of β -carotene may be related to a direct free radical scavenger activity of the molecule, and possible metabolites, mainly retinoic acid, generated by cells.

Angiogenesis is a crucial event in the remodeling of tissues of a growing body in embryonic and adult life, female ovulatory cycle, wound healing, tissue ischemia, and inflammatory processes as well as tumor malignancy [1].

Several pathways, including retinoid signaling, have been implicated in the development of the cardiovascular system in the fetal period. Nutritional deficiency of retinoids, RXR/RAR- or Raldh2-knock-out mice, are characterized by multiple developmental malformations, including severe cardiovascular defects and lack of ophthalmo-mesenteric vessels with disrupted formation of extra-embryonic vessels. However, cellular mechanisms, through which carotenoids/retinoids participate in the assembly of mammalian blood vessels, have not been defined.

Using Raldh2^{-/-} mice, Lai et al. documented that retinoic acid induced the expression of p21 and p27, the Cip/Waf family of Cdk inhibitors controlling cell cycle progression in ECs. Treatment of normal ECs with retinoids had no effect on the expression of cyclins (A, B, D3, and E), and Cdc2 or Cdk2. The same authors demonstrated, using Western blotting, that the formation of cyclin D1 (or D2) and Cdk4 complex was significantly reduced, while those of RA-induced p21 (or p27) and Cdk4, and cyclin D1 and D2 was greatly enhanced in the presence of retinoic acid. Reduced Cdk/cyclinD complex formation resulted in lower levels of phosphorylated retinoblastoma protein (Rb) in RA-treated ECs. Thus, there was no effect in terms of the number of cells (proliferation as similarly observed in our study), but there was significant decrease in the proportion of endothelial cells in phase S and increase in the proportion of cells in phase G1 with no evidence of apoptosis in ECs [57]. We have not observed any measurable effect of β -carotene on EC proliferation or apoptosis across the concentration range we used. Results of the microarray experiments showed weak β -carotene-induced up-regulation of key molecules participating in cell cycle regulation, such as MCM-5, polo-like kinase Plk1, or NUCKS controlling the G2/M checkpoint.

Although the analysis of the microarray data revealed strong (6.5-fold) β -carotene-induced up-regulation of the apoptosis facilitator Bcl2L11, a member of the Bcl2 family, we did not observe any changes in the rate of apoptosis in the cells as measured by the caspase activity. The direction of the surviving expression change observed in the microarray expression data was in accordance with the qRT-PCR results.

Thus, the proangiogenic activity of β -carotene in HUVECs, and possibly EPCs, seems to be unrelated to proliferation (or inhibition of apoptosis) [21], but associated with potent activation of chemotaxis and changes in the expression of genes mediating cell adhesion and matrix assembly. The results of the microarray experiments in our model strongly support this hypothesis. We demonstrated changes in the expression of several genes coding for proteins playing important roles in cell/cell and cell/matrix adhesion, matrix proteins and proteases, which regulate cell/matrix interaction during angiogenesis [1,7]. The list of the β -carotene-responsive genes includes those coding for proteins participating in cell/cell adhesion (VCAM1, SELP, CD24), cadherins (CELSR1), and catenins (CTNNA1L, CTNNB1). Expression of these genes was mainly down-regulated by β -carotene. The down-regulation of VCAM-1, ICAM, and selectin E by carotenoids, including β -carotene, in human aortic endothelium stimulated with Il-1 β was suggested to be responsible for modulation of the inflammatory response, and may prove the anti-inflammatory, protective effect of β -carotene on endothelium.

The expression of gene coding for proteins associated with cell-extracellular matrix adhesion, such as integrins (ITGA6) and SCARB1, was up-regulated by β -carotene. Members of extracellular ma-

trix-degrading enzymes involved in the regulation of matrix degradation and promotion of cell migration, such as MMP14, and the tissue plasminogen activator (tPA) gene (PLAT), were similarly up-regulated, while MMP12 was down-regulated. The expression of desintegrins, such as ADAMTS1, responsible for receptor shading, were also inhibited. The integrin-mediated stimulation of chemotaxis by the metalloproteinase mediated matrix degradation products is an important step of the capillary network formation [22,23].

The β -carotene-treated cells also showed the down-regulation of gene coding for various extracellular matrix components, such as collagens, fibrillin 1, laminin β 1, matrilin 2, and matrix Gla protein. The importance of the expression of cell surface proteins, which participate in cell/matrix (e.g., integrins) and cell/cell (e.g., cadherins, catenins, endoglin, ephrins, and their receptors) interactions and are required for regulation of proliferation, migration, and differentiation during angiogenesis, has been well documented [8,12,24,25]. Additionally, β -carotene up-regulated the expression of IL-8, a potent activator of EC migration [16], which was confirmed in this study by real-time PCR and Western blotting.

Remodeling of the extracellular matrix proteins by enzymatic degradation and synthesis of proteoglycans changes the extracellular matrix composition and regulates the migration of endothelial cells. Migration and cellular shape change are also associated with the stress fiber formation and reorganization of the cellular cytoskeletal proteins [26]. The involvement of nitric oxide in the chemotactic activity and cytoskeleton reorganization of endothelial cells was also reported [27]. The microarray experiments also showed the induction of endothelial nitric oxide synthase (eNOS) gene expression by β -carotene in HUVECs [24,25].

The G-protein binding receptors and regulators of Rho GTPases were the second largest group of genes, whose expression was significantly changed by β -carotene. The genes up-regulated by β -carotene, such as CXCR4 and IL-8 (whose induction was confirmed by real-time PCR in our study), as well as some others, such as GPR12, which was recently shown to be activated by sphingosine 1-phosphate (S1P)-Edg class receptor [27,28], DTR-heparan-binding epidermal growth factor (EGF)-like receptor, and the integrin alpha 6 (ITGA6) are known regulators of HUVEC migration that activate Rho GTPases [29]. Proangiogenic factors, such as cytokine receptors (VEGF, PDGF), stromal derived factor (SDF-1), IL-8, or S1P, lead to the activation of Rho/Rac/CDC42 small GTPases through the activation of G-protein-coupled receptors and by the interaction with adhesion-mediated signaling [15,16]. Rho GTPases regulate cytoskeletal changes responsible for cell motility, shape, and contraction. Rho GTPases promote actin-myosin interactions and contraction of the cell through the regulation of phosphorylation of the myosin light chains (MLCs). Our microarray data supported the hypothesis, that β -carotene leads to the activation of endothelial cells and migration of their progenitors, and, thus, proangiogenic activity, through the regulation of expression of the Rho/Rac/Cdc42 pathway.

Despite evidence of proangiogenic activity, β -carotene did not influence the tubulogenic activity per se in an in vitro 3D matrigel model. Lai et al. reported that RA deficiency had no effect on immature endothelial cell differentiation as evidenced by the expression of VEGF, Flk1-receptor, VE-cadherin, or angiopoietin Tie2 receptor [21]. On the contrary, Gaetano et al. [20] demonstrated that retinoids stimulate bFGF-dependent tubule formation by bovine ECs suspended in matrigel.

The recruitment and chemotaxis of endothelial progenitors to the niches of the damaged tissue is an essential process in the tissue reconstruction [30]. We have demonstrated that β -carotene used in the physiological concentration ranged found in human blood is a potent activator of chemotaxis of early endothelial progenitor cells, which is accompanied by changes in the expression of genes mediating cell adhesion and homing, though it does not activate the final markers of the endothelial differentiation. The activation of genes that play key roles in homing (CXCL12/SDF1) [31] and chemotaxis in angiogenesis (IL-8) [16] supports the observed chemotactic activity of β -carotene in EPCs. Interleukin-8 (IL-8) is a member of the family of 13 human CXC chemokines [32].

In spite of the fact that the cell fate determinant *Jagged-1* and *Notch-4* genes were down-regulated, which is an argument for the promotion of the differentiation [33], the effect of β -carotene on the vWF expression and eNOS, markers of endothelial cell maturation [34], was almost none. The lack of β -carotene influence on the tubulogenesis in the in vitro 3D matrigel model is consistent with the lack of activation of EPC differentiation by β -carotene.

Another clue for the incomplete differentiation of EPCs into endothelial cells was the lack of the PECAM1 up-regulation in response to β -carotene (data not shown [24,25]). It was demonstrated that PECAM1-induced cell/cell interactions are necessary for the organization of endothelial cells into tubules. Moreover, PECAM1 was also shown to be essential for the bFGF-induced angiogenesis [35].

The stimulation of CD36 and thrombospondin gene expression by β -carotene may also help explain the lack of tubulogenic activity of β -carotene, since activation of CD36 by TSP-1 was found to inhibit angiogenesis [36].

β -Carotene, similarly to other carotenoids, is a potent scavenger of free radicals, protecting against cell damage during oxidative stress [37]. The down-regulation of *BAX*, and up-regulation of *HO-1* genes by β -carotene ([38], this issue) confirm the protective activity of β -carotene toward endothelial progenitors. Hem oxygenase 1 shows protective properties against the hem-mediated oxygen-radical cellular stress. It also exerts anti-inflammatory effects and was shown to be involved in angiogenesis.

In summary, we postulate that the originally observed proangiogenic activity of β -carotene is related to the activation of chemotaxis of endothelial cells as well as endothelial progenitors. The effect of β -carotene on gene expression assessed by the gene expression microarrays is moderate. However, we observed changes in expression of genes supporting the involvement of β -carotene in the regulation of the extracellular matrix and adhesion molecules synthesis, resulting in a potent activation of Rho/Rac/Cdc42 GTPase signaling pathway, which in turn may promote the migration and in vivo formation of immature capillary networks by endothelial cells and their progenitors.

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