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Journal of Nutritional Biochemistry 35 (2016) 37–47

Journal of Nutritional Biochemistry

Blueberry inhibits invasion and angiogenesis in 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral squamous cell carcinogenesis in hamsters *via* suppression of TGF-β and NF-κB signaling pathways☆

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Received 5 February 2016; received in revised form 10 May 2016; accepted 7 June 2016

Abstract

Aberrant activation of oncogenic signaling pathways plays a pivotal role in tumor initiation and progression. The purpose of the present study was to investigate the chemopreventive and therapeutic efficacy of blueberry in the hamster buccal pouch (HBP) carcinogenesis model based on its ability to target TGF-β, PI3K/Akt, MAPK and NF-κB signaling and its impact on invasion and angiogenesis. Squamous cell carcinomas were induced in the HBP by 7,12-dimethylbenz[a]anthracene (DMBA). The effect of blueberry on the oncogenic signaling pathways and downstream events was analyzed by quantitative real-time PCR and immunoblotting. Experiments with the ECV304 cell line were performed to explore the mechanism by which blueberry regulates angiogenesis. Blueberry supplementation inhibited the development and progression of HBP carcinomas by abrogating TGF-β and PI3K/Akt pathways. Although blueberry failed to influence MAPK, it suppressed NF-κB activation by preventing nuclear translocation of NF-κB p65. Blueberry also modulated the expression of the oncomiR miR-21 and the tumor suppressor let-7. Collectively, these changes induced a shift to an anti-invasive and anti-angiogenic phenotype as evidenced by downregulating matrix metalloproteinases and vascular endothelial growth factor. Blueberry also inhibited angiogenesis in ECV304 cells by suppressing migration and tube formation. The results of the present study suggest that targeting oncogenic signaling pathways that influence acquisition of cancer hallmarks is an effective strategy for chemointervention. Identification of modulatory effects on phosphorylation, intracellular localization of oncogenic transcription factors and microRNAs unraveled by the present study as key mechanisms of action of blueberry is critical from a therapeutic perspective.

Keywords: Angiogenesis; Blueberry; Chemoprevention; Invasion; NF-κB; TGF-β

1. Introduction

Dysregulated intracellular signaling is recognized to play a pivotal role in cancer initiation and progression. In particular, transforming growth factor-β (TGF-β), nuclear factor-κB (NF-κB) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are among the most dysregulated intracellular signaling pathways in diverse malignancies [1–3].

TGF-β, a pleiotropic cytokine, plays a pivotal role in a plethora of biological processes including embryonic development, tissue homeostasis, immune regulation and inflammation [4]. Phosphorelay from the serine threonine kinase receptors TGFβR1 and TGFβR2 to downstream Smad transcription factors is the key to signaling in the canonical pathway. The phosphorylated R-smads (Smad-2 and Smad-3) associate with the common mediator Smad-4 with consequent nuclear translocation and transactivation of target genes. TGF-β signaling is negatively controlled by inhibitory Smad-7 [5]. TGF-β also evokes cellular responses through non-Smad pathways such as MAPK, PI3K/Akt and other factors [6].

Several upstream kinases including MAPK and PI3K/Akt are known to activate NF-κB by inducing the phosphorylation and degradation of the inhibitory subunit IκB-α followed by nuclear translocation of NF-κB and transactivation of target genes [7]. Aberrant activation of TGF-β, NF-κB and upstream kinase signaling pathways has been documented to promote epithelial-to-mesenchymal transition (EMT) and angiogenesis [8–10]. Mounting evidence has also revealed the critical role of the microRNAs oncomiR miR-21 and tumor suppressor let-7 in regulating tumor invasion and angiogenesis [11,12].

Invasion, a key event in metastasis, involves degradation of the extracellular matrix by the zinc-dependent matrix metalloproteinases (MMPs). MMPs are negatively regulated by tissue inhibitor of metalloproteinases (TIMPs) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) [13,14]. MMPs promote tumor angiogenesis through the generation of vascular endothelial growth factor (VEGF), a proangiogenic molecule that enhances

☆ Statement of conflict of interest: The authors state no conflict of interest.

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http://dx.doi.org/10.1016/j.jnutbio.2016.06.002
0955-2863/

neovascularization. In addition, VEGF is secreted by hypoxia-inducible factor-1α (HIF-1α) under hypoxic conditions that prevail in tumor cells [15].

Targeting oncogenic signaling pathways by dietary agents has evolved as a promising strategy in cancer prevention and treatment. Recently, dietary phytochemicals present in fruits especially berries also called "super fruits" have received significant attention owing to their innumerable health benefits [16]. Berries are readily available either fresh or as individually quick frozen fruit, juice, jams, jellies and snack foods [17]. In particular, blueberries from the flowering plants of the genus *Vaccinium* have been reported to exhibit potent antioxidant, anti-inflammatory and anti-carcinogenic effects owing to their rich content of anthocyanins and polyphenols [18]. Several studies have demonstrated that blueberry extract and powder inhibit tumorigenesis by blocking cellular processes involved in the acquisition of cancer hallmarks [19–21]. Previously, we reported a positive correlation between the chemopreventive potential of blueberry and its ability to induce Nrf2/Keap1 signaling [22].

The present study was aimed to investigate the anti-cancer effects of blueberry based on its ability to target TGF-β, PI3K/Akt, MAPK and NF-κB as well as the consequent downstream events invasion and angiogenesis. For this purpose, we used the 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis model. The buccal pouch of the hamster is an ideal organ to analyze anatomical and functional vascular changes during carcinogenesis and chemointervention [23–25]. In this study, we evaluated the chemopreventive and therapeutic efficacy of blueberry in the HBP model based on modulation of canonical TGF-β/Smad signaling; MAPK, PI3/Akt and NF-κB signaling; and the impact on invasion and angiogenesis by quantitative real-time PCR (qRT-PCR) and western blot analysis. In addition, experiments with the ECV304 cell line were performed to demonstrate the effect of blueberry in suppressing hypoxia-induced angiogenesis.

2. Materials and methods

2.1. Chemicals

Acrylamide, bovine serum albumin, bromophenol blue, DMBA, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), *N,N,N′,N′*-tetramethylene diamine (TEMED) and Trizol were purchased from Sigma Chemical Company, St. Louis, MO, USA. Blueberry powder containing 3.6 mg vitamin C and 102 mg anthocyanins per 3 g of powder was procured from Superfruit, Sweden. Power SYBR Green PCR master mix was obtained from Applied Biosystems, California, USA. Antibodies for GAPDH, TGFβR1, TGFβR2, Smad-2, p-Smad2/3$^{Ser465/423}$, Smad-4, Smad-7, NF-κB p50, NF-κB p65, p-NF-κB p65^{Ser536}, IκB-α, p-IκB^{Ser32}, IKKβ, Akt, p-Akt^{Ser473}, PI3K, ERK, p-ERK^{Thr202}, MMP-2, MMP-9, TIMP-2, RECK, HIF-1α, VEGF, VEGFR2 and pVEGFR2^{Tyr1175} were purchased from Santa Cruz Biotechnology, USA. p-Smad-2/Smad-3$^{Ser465/423}$, p-Akt^{Thr308}, p-NF-κB p65^{Ser536} and p-VEGFR2^{Tyr1175} ELISA kits were purchased from Cell Signaling Technology, USA. All other reagents used were of analytical grade.

2.2. Animals and ethics statement

Eight- to ten-week-old male Syrian hamsters weighing between 100 and 110 g were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition, India. The animals were housed three to a cage and provided with standard pellet diet and water *ad libitum*. The protocols for the experiments were approved by the Institutional Animal Ethics Committee and conducted according to the guidelines laid down by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

2.3. Experimental design

2.3.1. Experiment 1

The animals were randomized into experimental and control groups and divided into 6 groups of 6 animals each. In group 1, the right buccal pouches of hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks [22]. Hamsters in groups 2, 3 and 4 received in addition to DMBA, a basal diet containing 50, 100 and 200 mg/kg bw of blueberry, respectively. Group 5 animals were fed blueberry (200 mg/kg bw) alone for 14 weeks [26]. Group 6 animals received basal diet alone and served as an untreated control. The experiment was terminated at 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. The buccal pouch tissues were immediately subdivided and processed for further analyses.

2.3.2. Experiment 2

The animals were randomized into experimental and control groups and divided into 4 groups of 6 animals each. In group 1, the right buccal pouches of hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 12 weeks. Group 2 animals received, in addition to DMBA, a basal diet containing 200 mg/kg bw of blueberry from 12th week to 18th week. Group 3 animals were supplemented with blueberry (200 mg/kg bw) alone from 12th week until 18th week. Group 4 animals received basal diet alone and served as an untreated control. The experiment was terminated after 18 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. The buccal pouch tissues were immediately subdivided and processed for further analyses.

2.3.3. Experiment 3

ECV304 cells were cultured in DMEM basal medium with 10% fetal bovine serum and antibiotics. Confluent cultures of ECV304 cells were subcultured and maintained in CO_2 incubator at 37°C and 5% CO_2. For matrigel assay, the cells were maintained in MCDB basal medium with 2% fetal bovine serum. To subject cells to hypoxic conditions, ECV304 cells were incubated in a hypoxia chamber with 1% O_2.

2.4. RNA extraction and qRT-PCR

Total RNA from the buccal pouch tissues was extracted using Trizol reagent as described by Chomczynski and Sacchi in 1987 [26]. The RNA concentration was determined from the optical density at a wavelength of 260 nm (using an OD_{260} unit equivalent to 40 μg/ml of RNA). Five micrograms of isolated total RNA was reverse-transcribed to cDNA in a reaction mixture containing 4 μl of 5× reaction buffer, 2 μl of dNTP mixture (10 mM), 20 units of RNase inhibitor, 200 units of avian myeloblastosis virus reverse transcriptase and 0.5 μg of oligo(dT) primer (Promega, Wisconsin, USA) in a total volume of 20 μl. The reaction mixture was incubated at 42°C for 60 min and the reaction was terminated by heating at 70°C for 10 min. The cDNA was stored at −80°C until further use.

qRT-PCR was performed using Power SYBR Green master mix according to the manufacturer's instructions using a StepOne Plus thermocycler (Applied Biosystems). To the 1× PCR master mix, 2.5 μl of cDNA was added in a final volume of 20 μl. The PCR conditions were as follows: 95°C for 5 min, 40 cycles of 30 s at 95°C, 30 s at 52–60°C (based on the target) and 60 s at 72°C. Relative quantitative fold change compared to control was calculated using the comparative Ct method.

2.5. miRNA isolation

miRNA was isolated by miRNeasy minikit method as per manufacturer's instructions and quantified at 260 and 280 nm using a Biophotometer. cDNA was synthesized by Ncode VILO miRNA cDNA synthesis kit method following manufacturer's instructions. miRNA expression levels were quantified using StepOne Plus thermocycler (Applied Biosystems).

2.6. Western blotting

The buccal pouch tissue (150 mg) was homogenized using lysis buffer containing 62.5 mM Tris (pH 6.8), 10 mM Hepes (pH 7.9),10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, protease inhibitor cocktail and 10% SDS. The homogenate was centrifuged at 14,000*g* at 4°C for 20 min. Nuclear and cytoplasmic extracts were prepared as described by Legrand-Poels *et al.* [27]. SDS-PAGE was performed using equivalent protein extracts (50 μg) from each sample. The resolved proteins were electrophoretically transferred to nitrocellulose membrane. The membranes were then incubated for 1 h in 1× PBS containing 5% non-fat dry milk to block nons-pecific binding sites and probed with primary and secondary antibodies as per manufacturer's instructions. After washing with high and low salt buffers, the protein bands were visualized using enhanced chemiluminescence. Densitometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

2.7. ELISA

The levels of p-Smad-2/Smad-3$^{Ser465/423}$, p-Akt^{Thr308}, p-NF-κB p65^{Ser536} and pVEGFR2^{Tyr1175} were determined using Sandwich ELISA kit (Cell Signaling Technology, USA) according to the manufacturer's instructions.

2.8. Microvascular density (MVD)

MVD was analyzed by immunohistochemical staining with anti-CD34 antibody. Areas of highest neovascularization were located and the images were captured in a minimum of five different fields. Microvessels were counted by two independent investigators and the data were represented as the number of vessels per field of view.