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ORIGINAL ARTICLE

β-carotene Regulates the Murine Liver Microenvironment of a Metastatic Neuroblastoma

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Background: The anticarcinogenic effects of β-carotene (BC) have been well-characterized. However, the effect of BC on the microenvironment of a tumor remains to be investigated, especially since normal tissue proximal to a tumor has been shown to play a critical role in cancer progression and metastasis. For young children, neuroblastoma (NB) is the most common extracranial solid cancer diagnosed. Therefore, in the present study, effect of BC on the murine liver microenvironment of a metastatic NB was evaluated.
Methods: Using a mouse model, three experimental groups were established: control mice, mice receiving an injection of SK-N-BE(2)C cells (TC), and mice receiving an injection of SK-N-BE(2)C cells plus 2 mg/kg BC twice a week (BC). Eight weeks after the injection of tumor, liver tissues were collected from all three groups, with the TC and BC tissues collected proximal to the metastatic NBs.
Results: Compared to control tissues, BC tissues exhibited lower levels of proliferation, apoptosis, and metastasis. Assays for these processes included the detection of lower levels of proliferating cell nuclear antigen (PCNA), Bax, MMP2, and MMP9. In addition, higher levels of Bcl-2 were detected. Fewer cells undergoing an epithelial mesenchymal transition (EMT) were also observed in the BC group. Furthermore, BC tissues were associated with reduced expression of cancer stem cell marker, delta-like 1 homologue (DLK1), lower levels of *VEGF* mRNA and fewer CD31-positive cells. Finally, The antioxidant capability of the tumor microenvironment for the BC group was enhanced with higher expression levels of glutathione peroxidase (GPX), catalase, and manganese superoxide (MnSOD) detected.
Conclusion: These data suggest that BC affects the microenvironment of a tumor, and this enhances the anti-cancer effects of BC. **(J Cancer Prev 2013;18:337-345)**

Key Words: β-carotene, Microenvironment, Metastasis, Neuroblastoma

INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid cancer diagnosed in young children. NBs often exhibit unexpected clinical behaviors, including spontaneous regression, maturation, or aggressive progression.[1,2] For stage 4 NB patients that are older than one year, these patients typically have a poor outcome, with 5-year survival rates of 30-40%.[3] In addition, more than half of these children initially present with nonresectable tumors that have undergone disseminated metastasis to distant organs. The most sites of metastasis include bone marrow, liver, and the non-contiguous lymph nodes. For children with NB bone metastasis, the mortality rate is greater than 90%.[4,5]

Metastasis is a complex multistep process that results in the ability of tumor cells to colonize in other regions of the body.[6] During this process, the interaction of tumor cells with their microenvironment at the site of metastasis represents a key step.[7] In recent studies, both tumor cells and host cells of a tumor microenvironment were found to contribute to tumor metastasis.[6] Correspondingly, there

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are various signaling molecules which are secreted in an autocrine and paracrine fashion by a variety of cells present in the tumor microenvironments, and these molecules promote the proliferation, metastasis, and epithelial-to-mesenchymal transition (EMT) of affected cells. In combination, these are critical biologic processes that are altered during metastasis.[8] While proliferation and apoptosis are fundamental processes in the early stages of carcinogenesis, they also represent vital markers for the characterization of histologically normal tissues, and can also be used to evaluate neoplastic growth potential.[9] Cell invasion and migration are also key steps, with cancer cells degrading the underlying basement membrane in order to detach from the primary tumor.[10] To migrate through the extracellular matrix (ECM), cancer cells use matrix metalloproteinase (MMP) enzymes. These zinc-binding endopeptidases exhibit proteolytic activity and facilitate the invasion and migration of tumor cells by degrading the ECM.[11,12] Thus, MMPs represent targets for the inhibition of tumor spread.

The EMT is a unique process which epithelial cells lose cell-to-cell contacts and transform into cells with mesenchymal characteristics.[13] Furthermore, the EMT process is related to cancer progression that involves metastasis. Factor produced by the microenvironment, including various growth factors, can direct EMT-mediated tumor metastasis.[14] Furthermore, accumulating evidence indicates that tumor-initiating cells, termed cancer stem cells (CSCs), are dependent on the microenvironment for their formation. A complex network of crosstalk exists between CSCs and their environment, with growth factors, hormones, and the ECM promoting CSC traits.[15] Several studies have further reported that CSCs are resistant to conventional therapies, are associated with a poor prognosis, and exhibit a high migratory potential. These characteristics are consistent with the proposed role for CSCs in tumor cell invasion and metastasis.[16] Currently, CSC niches remain incompletely characterized, although components of the tumor microenvironment have been shown to regulate the characteristics of CSCs.[17] The induction of angiogenesis is an important early stage in the growth of tumors. Tumor angiogenesis through secreting many tumor-derived soluble factors like most notably vascular endothelial growth factor (VEGF) is a key step of metastasis.[18] Increased vascularity is hypothesized to contribute to the poor prognosis of a tumor,[19] while the vascular endothelium may also affect the formation of pre-metastatic and metastatic niches.[20]

Reactive oxygen species (ROS) also play an important role in the carcinogenesis and malignant progression of tumor cells.[19] For example, by inducing inflammation/repair and angiogenesis, ROS can promote cell motility and a permissive state for a tumor microenvironment.[19] Correspondingly, a strong relationship between dietary antioxidants or antioxidant enzymes, and anti-cancer effects have been reported.[19] In particular, β-carotene (BC) is a well-known antioxidant and precursor to vitamin A that is found in many fruits and vegetables that are green or orange in color.[21] In observational studies, a high dietary intake of fruits and vegetables rich in BC has been associated with a reduced risk for various cancers. Moreover, in experimental studies, BC was found to regulate the proliferation and apoptosis of several cancer cell lines.[22,23] Although the anti-cancer effect of BC is well-characterized, the effect of BC on the microenvironment of a metastatic tumor remains to be investigated. Therefore, in the present study, the effects of BC on murine liver tissue proximal to a metastatic NB were investigated.

MATERIALS AND METHODS

1. Cell culture and reagents

A human NB cell line, SK-N-BE(2)C (BE(2)C), was purchased from American Type Culture Collection (ATCC, Rockville, MD) and was maintained in a 1:1 mixture of Minimum Essential Medium (MEM, Welgene, Daegu, Korea) and Ham's F-12 medium (Welgene, Daegu, Korea) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) at 37°C with 5% CO_2. BC was purchased from Sigma Chemical Co. (St. Louis, MO).

2. Animal treatment

Five-week-old male BALB/c nu/nu mice (Central Lab, Animal Inc., Seoul, Korea) were maintained under specific pathogen-free conditions (College of Pharmacy, Ewha Womans University, Seoul, Korea) at 25±2°C, 65±5%

humidity with 12 h/12 h light/dark cycles. Animals received pellet diets and water *ad libitum*. Three experimental groups were established: control mice (Ctrl), mice that received an injection of BE(2)C cells (TC), and mice that were administered 2 mg/kg b.w. BC twice a week for 20 days, then were injected with BE(2)C cells (BC). There were ten mice per group. The Ctrl group received an oral administration of corn oil as a vehicle twice a week throughout the experimental period. The BC group received BC as indicated for 20 days prior to the injection of 2.0×10^6 BE(2)C cells intravenously via the lateral tail vein on day 21. The TC mice were injected with BE(2)C cells in parallel. Eight weeks later, all of the mice from the three groups were sacrificed. Animal care and experimental protocols for this study were approved by the Animal Care and Use Committee of Ewha Womans University (IACUC approval NO:2012-01-014). Tumor volume was estimated using the following formula: $V= W^2\times L\times 1/2$, where W represents tumor width and L represents tumor length.

3. Histology and Immunohistochemistry

Tissues were fixed in 4% neutral buffered formaldehyde for 24 h then were paraffin embedded. Sections were cut (5 μM) and fixed with acetone and chloroform and then were analyzed by immunohistochemistry. Briefly, endogenous peroxidases were inactivated in section with an incubation in 3% H_2O_2 in PBS. Sections were subsequently incubated with a protein-blocking solution for 20 min, then with primary antibodies directed against CD31 (Abcam, Cambridge, UK) overnight at 4°C. Sections were incubated with peroxidase-conjugated secondary antibodies (Envision kit, DAKO, Ely, UK) at RT. After 1 h, sections were stained with 3,-3'-diaminobenzidinetetrahydrochloride (DAB) and counterstained with hematoxylin for 1 min. Slides were mounted using Permount mounting medium (Fisher Scientific, Pittsburgh, PA).

4. Western blot analysis

Frozen liver tissues were weighed and homogenized in ice-cold PRO-PREP protein extraction solution (Intron Biotechnology, Seoul, Korea). Homogenates were centrifuged (12000×g, 15 min, 4°C) and supernatants were collected and stored at −80°C. Denatured tissue homogenates were separated using gradient (8-12%) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes and were blocked with 5% skim milk in Tris-buffered saline containing Tween-20 (TBST) buffer. Membranes were incubated with specific primary antibodies raised against proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin (Cell Signaling Technology, Beverly, MA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Bax (Santa Cruz Biotechnology, Santa Cruz, CA), glutathione peroxidase (GPX, Abfrontier, Seoul, Korea), manganese superoxide disumutase (MnSOD, Stressgen Biotechnologies, Victoria, Canada), catalase (Abfrontier, Seoul, Korea), and anti-α-tubulin (Sigma Aldrich, St. Louis, MO) overnight at 4°C. After being washed three times with TBST, membranes were incubated with the appropriate secondary antibodies at RT. After 1h, membrane-bound antibodies were detected using an enhanced chemiluminescence reagent (Animal Genetics Inc. Suwon, Kyonggi-do).

5. Reverse transcription-PCR and quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA), precipitated with isopropanol, and the resulting RNA pellet was washed in 75% ethanol. cDNA was synthesized by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). PCR amplification included an initial incubation at 94°C for 5 min, followed by denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s using Taq polymerase (TAKARA, Tokyo, Japan). The resulting PCR products were separated using a 2% agarose gel containing ethidium bromide. The primers used included: 5'-ACTGGACCCTGGCTTTACTG-3' (forward) and 5'-TCTGCT CTCCTTCTGTCGTG-3' (reverse) for *VEGF*; 5'-AGCACGTG TACAGACACTG GTC-3' (forward) and 5'-ATCCTTGGTCAGGACAGAAGCC-3' (reverse) for *MMP2*; 5'-AAGGAC GGCCTTCTTCTGGCA CACGCCTTT-3' (forward) and 5'-GTGGRATAGTGGGACACAT AGTGG-3' (reverse) for *MMP9*. All samples were normalized to the housekeeping gene, β-actin, which was not affected by the experimental treatments.

6. Statistical analysis

Results are presented as the mean±standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test were performed using GraphPad PRISM software (GraphPad Software, SanDiego, CA). A P-value less than 0.05 was considered statistically significant.

RESULTS

1. BC reduces tumor volume and inhibits the proliferation and apoptosis of normal liver tissues proximal to metastatic NB

For Ctrl, TC, and BC experimental mouse group, no difference in body weight was observed during the experimental period (data not shown). At the time of sacrifice, only the TC group had developed metastatic tumors in liver tissues. In addition, treatment with BC resulted in a 92% decrease in NB volume for the BC group compared to the TC group.[24]

Previously, it was reported that high levels of PCNA were detected in normal tissues that surrounded tumors.[25] To understand the effect of BC on cell proliferation in the tumor microenvironment, liver tissues were collected from all three groups, with the TC and BC tissues collected proximal to the metastatic NBs. Compared with the control group, higher levels of PCNA were detected for the TC tissues compared to the BC tissues (Fig. 1A). This observation suggests that BC inhibits cell proliferation in the normal tissue that surrounds the tumor, thereby providing an unfavorable environment for tumor metastasis.

BC has been shown to induce apoptosis in many types of tumor cells,[26,27] yet not in normal cells.[28] When expression of Bcl-2, an anti-apoptotic protein, was assayed by western blot in the present study, lower levels of Bcl-2 were detected in the TC samples compared to the control samples. In contrast, higher levels of Bcl-2 were detected in BC samples (Fig. 1B). Levels of Bax, a pro-apoptotic protein, were also assayed. Consistent with the Bcl-2 results, higher levels of Bax were detected in the TC samples compared to the control samples, and this effects was inhibited in the presence of BC (Fig. 1B). Taken together, these results suggest that BC affects the levels of apoptosis in the tumor microenvironment of metastatic tumor.

2. BC suppresses metastasis by inhibiting MMPs, vimentin, and DLK1 in normal liver tissues proximal to metastatic NB

MMPs mediate several signaling pathways, including those that affect cell growth, inflammation, and angiogenesis during tumor progression.[29] Stromal cells also participate in tumor-stroma cross-talk, and this can lead to the secretion of MMPs and the degradation of the ECM, thereby facilitating metastasis.[30] To investigate the effect of BC on MMP secretion by stromal cells of a tumor microenvironment, expression of *MMP2* and *MMP9* were analyzed. The mRNA levels of both MMPs were found to be highly induced in the TC group. In contrast, both MMP levels were down-regulated in BC tissues (Fig. 2A). These data suggest that BC can inhibit metastasis by regulating the expression of MMPs in the tumor microenvironment.

The metastasis process involves changes in cancer cells and the tumor microenvironment.[31] The EMT is, also a

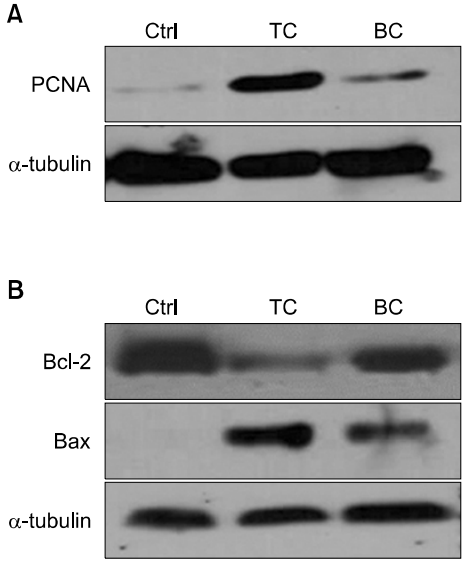


Fig. 1. BC inhibits the proliferation and apoptosis in normal liver tissues that are proximal to a metastatic NB. Western blotting was used to detect protein levels of PCNA (A), Bcl-2 and Bax (B). The α-tubulin was used as a loading control. Ctrl, control; TC, tumor control; BC, β-carotene supplementation.

process that promotes carcinoma invasion and metastasis with CSCs mediating tumor growth and metastasis.[32,33] To investigate whether the presence of BC affects the EMT and CSCs to inhibit metastasis, levels of vimentin, a marker of mesenchymal cells, and delta-like 1 homologue (DLK1), a CSC marker, were detected in normal liver tissues proximal to metastatic tumor. In the BC tissues, lower levels of both vimentin and DLK1 were detected following tumor injection compared to TC (Fig. 2B). Thus, BC may play a critical role in suppressing the EMT and the generation of CSCs in a tumor microenvironment.

3. BC inhibits angiogenesis proximal to a metastatic NB

VEGF is a key modulator of angiogenesis, a process which induces nearby vessels to support tumor growth.[34] To examine whether angiogenesis is inhibited in a tumor microenvironment in the presence of BC, levels of *VEGF* were analyzed using quantitative PCR. Higher levels of *VEGF* mRNA were detected in the TC group compared with the control group, while decrease in *VEGF* mRNA levels was observed for the BC group (Fig. 3A). In immunohistochemistry assays, stronger expression of the endo-

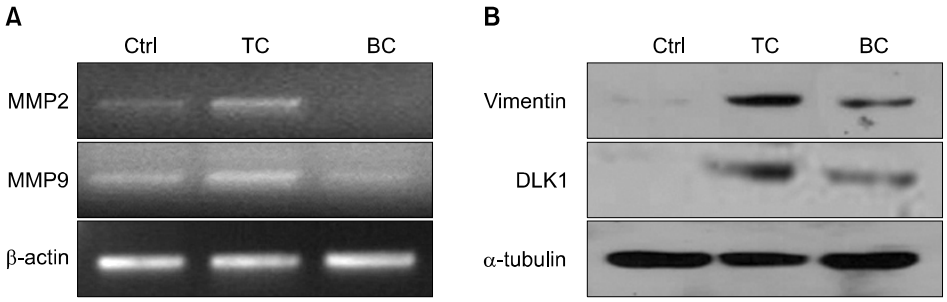
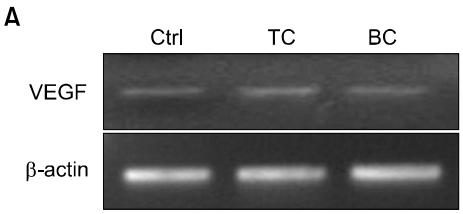


Fig. 2. BC inhibits tumor cell invasion by inhibiting MMPs, vimentin, and DLK1 in normal liver tissue proximal to a metastatic NB. (A) Using RT-PCR, mRNA levels of *MMP2* and *MMP9* were analyzed. Detection of β-acin was used as a loading control. (B) Levels of DLK1 and vimentin were detected using western blotting, and detection of α-tubulin was used as a loading control. Ctrl, control; TC, tumor control; BC, β-carotene supplementation.



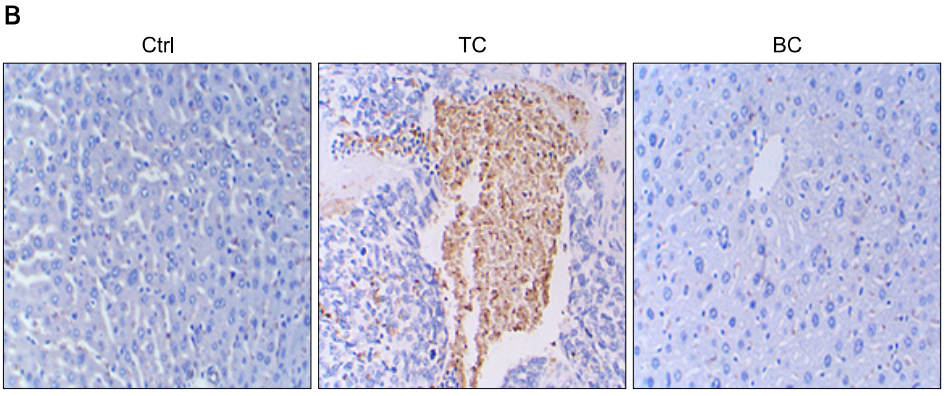


Fig. 3. BC inhibits angiogenesis in the liver near a metastatic NB. (A) Using RT-PCR, mRNA levels of *VEGF* were analyzed. (B) Expression of CD31 was detected immunohistochemically in sections of liver tissue obtained proximal to a metastatic NB (magnification 250×). Ctrl, control; TC, tumor control; BC, β-carotene supplementation.

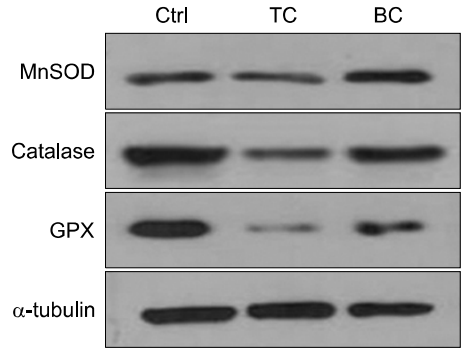


Fig. 4. BC enhances the anti-oxidant properties of liver tissues proximal to a metastatic NB. Levels of GPX, MnSOD, and catalase were detected by western blot. Levels of α-tubulin were detected as a loading control. Ctrl, control; TC, tumor control; BC, β-carotene supplementation.

thelial marker, CD31, was observed for TC tissue sections compared to control tissue sections. In contrast, fewer CD31-positive cells were observed in BC tissue sections (Fig. 3B). In combination, these results indicate that angiogenesis is inhibited in a tumor microenvironment in the presence of BC.

4. BC mediates antioxidant properties in a tumor microenvironment

ROS play a role in the carcinogenesis and malignant progression of tumor cells, including tumor invasion and angiogenesis.[19] BC is a well-known antioxidant,[21] and thus was investigated for its role in regulating oxidative stress in a tumor microenvironment. Specifically, the expression levels of several antioxidant enzymes were analyzed. For the TC group, lower levels of MnSOD, GPX, and catalase were detected compared to the control group (Fig. 4). In contrast, the BC group exhibited higher levels of these antioxidant enzymes compared to the TC group. These results indicate that BC mediates antioxidant effects on normal liver tissue proximal to a metastatic NB.

DISCUSSION

The aim of this study was to elucidate the effects of BC on normal murine liver tissue proximal to metastatic NB. Previous studies have shown that the tumor microenvironment is critical for tumorigenesis, angiogenesis, and metastasis.[35,36] It has also been hypothesized that a molecular marker of noncancerous liver tissue may serve as a factor for predicting cancer risk.[37] In the present study, the effects of BC on a tumor microenvironment were examined based on the antioxidant properties of BC that have been well-characterized, as well as the potential for BC to serve as a chemopreventive agent.

Treatment with BC resulted in lower levels of PCNA, a protein that mediates cell proliferation in non-proliferating liver cells. In contrast, higher levels of PCNA were detected for the TC group. These results were consistent with those of recent studies where higher levels of proliferation were detected in benign hepatic tissues proximal to a tumor.[38,39] Zeng et al. also reported that PCNA-positive cells were primarily distributed in the peripheral region of a tumor nest and also at the site of an invasive hepatocellular carcinoma.[40] Taken together, these results suggest that BC affects a tumor microenvironment by regulating cell proliferation.

Upadhyaya et al. have documented that BC serves as an apoptotic factor for cancer cells, yet not for normal cells.[28] However, in the present study, BC treatment was found to inhibit the apoptosis of normal liver tissue proximal to a metastatic NB, and this was associated with higher expression of Bcl-2, an anti-apoptotic protein, and reduced expression of Bax, a pro-apoptotic protein. In normal cells, a homeostatic balance between cell proliferation and apoptosis is needed to prevent carcinogenesis. Correspondingly, increase in proliferation has been found to correlate with an increase in apoptosis, and vice versa in normal cell.[41]

MMP2 and MMP9 regulate tumor microenvironments and are typically expressed at high levels. In particular, they accelerate the recruitment of macrophage and induce tumor metastasis and angiogenesis.[42] In the present study, mRNA levels of *MMP2* and *MMP9* were high in TC group tissues, and were significantly lower in BC tissues. Moreover, in the latter case, the tumor microenvironment became less permissive. Correspondingly, it has been reported that MMP 9 enhanced by the injection of cholesterol hydroxides (Chol-OOHs) into murine skin, and this change could be suppressed with the addition of BC to the diet.[43]

The EMT process allows epithelial cells to replace their epithelial characteristics with the traits of mesenchymal

cells.[44] The EMT not only induces tumor progression, but can also act as a tumor initiator and can affect a microenvironment to promote tumor formation.[44] *In vitro*, inhibition of vimentin expression has been shown to recover the epithelial characteristics of cells that have undergone an EMT.[45] Correspondingly, vimentin serves as a marker of the EMT and has also been linked to the metastatic potential of cancer cells.[32] In the present study, expression of vimentin was higher in the TC group compared to the BC group. These results suggest that BC suppress metastasis by reducing the mesenchymal characteristics of the liver tissues.

It has been hypothesized that CSCs that exist in hypoxic niches that are distal from blood vessels, and CSCs that exist in perivascular niches that may or may not be hypoxic, are affected by the specific microenvironment of a tumor.[17] DLK1 is a marker of CSCs, and knock-down of DLK1 has been shown to attenuate tumorigenecity and increase neuronal differentiation.[46] These results demonstrate the potential for DLK1 to serve as a therapeutic target. In the present study, BC supplementation resulted in lower levels of DLK1 expression in normal liver tissue adjacent to a metastatic NB. These results suggest that regulation of a tumor's microenvironment by BC is not compatible with CSC survival.

Expression of VEGF in the TC group was higher than that of the control group. These results are consistent with those of Zhuang et al. who also observed higher levels of VEGF expression, and a higher recruitment of endothelial progenitor cells, in nonmalignant liver tissues compared to tumor vessels.[47] In contrast, lower level of *VEGF* was associated with the BC tissues analyzed. Consistent with this data, immunohistochemistry analysis has shown that animals fed with BC showed diminished endothelial cell specific CD31 expression in the normal liver tissue proximal to tumor. Taken together, these findings support the hypothesis that BC down-regulates VEGF and suppresses angiogenesis in a tumor microenvironment.

When metastasizing tumor cells enter the systemic circulation, they suffer oxidative stress.[48] As a result, many of the tumor cells are damaged and ROS are generated. These ROS can activate angiogenesis and the secretion of MMP to induce metastasis.[49] In the present study, the antioxidant enzymes assayed were found to be expressed at lower levels in the TC group, and this was consistent with the results of a previous study where catalase activity in a tumor microenvironment was lower than that of normal tissue.[48] However, in the presence of BC, expression levels of MnSOD, GPX, and catalase were higher. Similarly, when catalase derivative was administered to mice following an injection of tumor cells, a reduced number of metastatic colonies were found in the liver.[50] Taken together, these data provide further evidence that BC has the capacity to improve the antioxidant status of a tumor microenvironment to negatively affect tumor metastasis.

In conclusion, the results of the present study demonstrate that BC can mediate an inhibition of proliferation, apoptosis, angiogenesis, and the EMT process in the microenvironment of a tumor. Furthermore, based on the assays performed, valuable insight into the mechanistic details of these processes was obtained and this enhances our understanding of the anti-carcinogenic effect of BC.

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REFERENCES

1. Asgharzadeh S, Salo JA, Ji L, Oberthuer A, Fischer M, Berthold F, et al. Clinical significance of tumor-associated inflammatory cells in metastatic neuroblastoma. J Clin Oncol 2012;30:3525-32.
2. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3:203-16.
3. Naranjo A, Parisi MT, Shulkin BL, London WB, Matthay KK, Kreissman SG, et al. Comparison of (1)(2)(3)I-metaiodobenzylguanidine (MIBG) and (1)(3)(1)I-MIBG semi-quantitative scores in predicting survival in patients with stage 4 neuroblastoma: a report from the Children's Oncology Group. Pediatr Blood Cancer 2011;56:1041-5.
4. Matthay KK, Villablanca JG, Seeger RC, Stram DO, Harris RE, Ramsay NK, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 1999;341:1165-73.
5. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY. Association of multiple copies of the N-myc on-

cogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111-6.
6. Zoccoli A, Iuliani M, Pantano F, Imperatori M, Intagliata S, Vincenzi B, et al. Premetastatic niche: ready for new therapeutic interventions? Expert Opin Ther Targets 2012;16 Suppl 2:S119-29.
7. Fidler IJ. The organ microenvironment and cancer metastasis. Differentiation 2002;70:498-505.
8. Liao Z, Boileau TW, Erdman JW Jr. Clinton SK: Interrelationships among angiogenesis, proliferation, and apoptosis in the tumor microenvironment during N-methyl-N-nitrosourea androgen-induced prostate carcinogenesis in rats. Carcinogenesis 2002;23:1701-11.
9. Ananthanarayanan V, Deaton RJ, Yang XJ, Pins MR, Gann PH. Alteration of proliferation and apoptotic markers in normal and premalignant tissue associated with prostate cancer. BMC cancer. 2006;6:73.
10. Yamaguchi H, Wyckoff J, Condeelis J. Cell migration in tumors. Curr Opin Cell Biol 2005;17:559-64.
11. Goldfarb A, King CL, Rosenzweig BP, Feit F, Kamat BR, Rumancik WM, et al. Cardiac lymphoma in the acquired immunodeficiency syndrome. Am Heart J 1989;118:1340-44.
12. Fingleton B. Matrix metalloproteinases: roles in cancer and metastasis. Frontiers in bioscience: a journal and virtual library 2006;11:479-91.
13. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442-54.
14. Creighton CJ, Gibbons DL, Kurie JM. The role of epithelial-mesenchymal transition programming in invasion and metastasis: a clinical perspective. Cancer Manag Res 2013;5: 187-95.
15. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer 2009;9:239-52.
16. Salnikov AV, Liu L, Platen M, Gladkich J, Salnikova O, Ryschich E, et al. Hypoxia induces EMT in low and highly aggressive pancreatic tumor cells but only cells with cancer stem cell characteristics acquire pronounced migratory potential. PloS One 2012;7:e46391.
17. Lin Q, Yun Z. Impact of the hypoxic tumor microenvironment on the regulation of cancer stem cell characteristics. Cancer Biol Ther 2010;9:949-56.
18. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. Nat Rev Cancer 2009;9:285-93.
19. Pani G, Galeotti T, Chiarugi P. Metastasis: cancer cell's escape from oxidative stress. Cancer Metastasis Rev 2010;29: 351-78.
20. Ding L, Sunamura M, Kodama T, Yamauchi J, Duda DG, Shimamura H, et al. In vivo evaluation of the early events associated with liver metastasis of circulating cancer cells. Br J Cancer 2001;85:431-8.
21. Lin J, Cook NR, Albert C, Zaharris E, Gaziano JM, Van Denburgh M, et al. Vitamins C and E and beta carotene supplementation and cancer risk: a randomized controlled trial. J Natl Cancer Inst 2009;101:14-23.
22. van Poppel G, Goldbohm RA. Epidemiologic evidence for beta-carotene and cancer prevention. Am J Clin Nutr 1995; 62(6 Suppl):1393S-402.
23. Palozza P, Serini S, Maggiano N, Angelini M, Boninsegna A, Di Nicuolo F, et al. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by beta-carotene through down-regulation of cyclin A and Bcl-2 family proteins. Carcinogenesis 2002;23:11-8.
24. Kim YS, Lee HA, Lim JY, Kim Y beta-carotene inhibits neuroblastoma cell invasion and metastasis in vitro and in vivo by decreasing level of hypoxia-inducible factor-1 alpha. J Nutr Biochem 2013:submitted.
25. Hall PA, Coates PJ, Goodlad RA, Hart IR, lane DP. Proliferating cell nuclear antigen expression in non-cycling cells may be induced by growth factors in vivo. Br J Cancer 1994;70:244-7.
26. Jang SH, Lim JW, Kim H: Mechanism of beta-carotene-induced apoptosis of gastric cancer cells: involvement of ataxia-telangiectasia-mutated. Ann N Y Acad Sci 2009;1171: 156-62.
27. Palozza P, Serini S, Torsello A, Di Nicuolo F, Maggiano N, Ranelletti FO, et al. Mechanism of activation of caspase cascade during beta-carotene-induced apoptosis in human tumor cells. Nutr Cancer 2003;47:76-87.
28. Upadhyaya KR, Radha KS, Madhyastha HK. Cell cycle regulation and induction of apoptosis by beta-carotene in U937 and HL-60 leukemia cells. J Biochem Mol Biol 2007;40: 1009-15.
29. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010;141:52-67.
30. Li H, Fan X, Houghton J. Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem 2007; 101:805-15.
31. Yilmaz M, Christofori G, Lehembre F. Distinct mechanisms of tumor invasion and metastasis. Trends Mol Med 2007; 13:535-41.
32. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 2008;14:818-29.
33. Reya T. Regulation of hematopoietic stem cell self-renewal. Recent Prog Horm Res 2003;58:283-95.
34. Kim Y, Stolarska MA, Othmer HG. The role of the microenvironment in tumor growth and invasion. Progress in Biophysics and Molecular Biology 2011;106:353-79.
35. Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer 2007;7:139-47.
36. Cheng JD, Weiner LM. Tumors and their microenvironments: tilling the soil. Commentary re: A. M. Scott et al., A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. Clin Cancer Res 2003;9:1639-47.
37. Utsunomiya T, Shimada M, Imura S, Morine Y, Ikemoto T, Mori M. Molecular signatures of noncancerous liver tissue can predict the risk for late recurrence of hepatocellular carcinoma. J Gastroenterol 2010;45:146-52.
38. Tarao K, Shimizu A, Harada M, Kuni Y, Ito Y, Tamai S, et al. Difference in the in vitro uptake of bromodeoxyuridine between liver cirrhosis with and without hepatocellular carcinoma. Cancer 1989;64:104-9.
39. Tarao K, Ohkawa S, Shimizu A, Harada M, Nakamura Y,

Ito Y, et al. Significance of hepatocellular proliferation in the development of hepatocellular carcinoma from anti-hepatitis C virus-positive cirrhotic patients. Cancer 1994; 73:1149-54.
40. Zeng WJ, Liu GY, Xu J, Zhou XD, Zhang YE, Zhang N. Pathological characteristics, PCNA labeling index and DNA index in prognostic evaluation of patients with moderately differentiated hepatocellular carcinoma. World J Gastroenterol 2002;8:1040-4.
41. Leoncini L, Del Vecchio MT, Megha T, Barbini P, Galieni P, Pileri S, et al. Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. Am J Pathol 1993;142:755-63.
42. Wu SD, Ma YS, Fang Y, Liu LL, Fu D, Shen XZ. Role of the microenvironment in hepatocellular carcinoma development and progression. Cancer Treat Rev 2012;38:218-25.
43. Minami Y, Kawabata K, Kubo Y, Arase S, Hirasaka K, Nikawa T, et al. Peroxidized cholesterol-induced matrix metalloproteinase-9 activation and its suppression by dietary beta-carotene in photoaging of hairless mouse skin. J Nutr Biochem 2009;20:389-98.
44. Trujillo KA, Heaphy CM, Mai M, Vargas KM, Jones AC, Vo P, et al. Markers of fibrosis and epithelial to mesenchymal transition demonstrate field cancerization in histologically normal tissue adjacent to breast tumors. Int J Cancer 2011;129:1310-21.
45. Paccione RJ, Miyazaki H, Patel V, Waseem A, Gutkind JS, Zehner ZE, et al. Keratin down-regulation in vimentin-positive cancer cells is reversible by vimentin RNA interference, which inhibits growth and motility. Mol Cancer Ther 2008;7:2894-903.
46. Kim Y, Lin Q, Zelterman D, Yun Z. Hypoxia-regulated delta-like 1 homologue enhances cancer cell stemness and tumorigenicity. Cancer Res 2009;69:9271-80.
47. Yu DC, Chen J, Sun XT, Zhuang LY, Jiang CP, Ding YT. Mechanism of endothelial progenitor cell recruitment into neo-vessels in adjacent non-tumor tissues in hepatocellular carcinoma. BMC Cancer 2010;10:435.
48. Nishikawa M, Hyoudou K, Kobayashi Y, Umeyama Y, Takakura Y, Hashida M. Inhibition of metastatic tumor growth by targeted delivery of antioxidant enzymes. J Control Release 2005;109:101-7.
49. Chua CC, Hamdy RC, Chua BH. Upregulation of vascular endothelial growth factor by H2O2 in rat heart endothelial cells. Free Radic Biol Med 1998;25:891-7.
50. Nishikawa M, Tamada A, Hyoudou K, Umeyama Y, Takahashi Y, Kobayashi Y, et al. Inhibition of experimental hepatic metastasis by targeted delivery of catalase in mice. Clin Exp Metastasis 2004;21:213-21.