β-Caryophyllene potently inhibits solid tumor growth and lymph node metastasis of B16F10 melanoma cells in high-fat diet–induced obese C57BL/6N mice

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Abstract

We reported previously that high-fat diet (HFD) feeding stimulated solid tumor growth and lymph node (LN) metastasis in C57BL/6N mice injected with B16F10 melanoma cells. β-caryophyllene (BCP) is a natural bicyclic sesquiterpene found in many essential oils and has been shown to exert anti-inflammatory activities. To examine whether BCP inhibits HFD-induced melanoma progression, 4-weeks old, male C57BL/6N mice were fed a control diet (CD, 10 kcal% fat) or HFD (60 kcal% fat + 0, 0.15 or 0.3% BCP) for the entire experimental period. After 16 weeks of feeding, B16F10s were subcutaneously injected into mice. Three weeks later, tumors were resected, and mice were killed 2 weeks post-resection. Although HFD feeding increased body weight gain, fasting blood glucose levels, solid tumor growth, LN metastasis, tumor cell proliferation, angiogenesis and lymphangiogenesis, it decreased apoptotic cells, all of which were suppressed by dietary BCP. HFD feeding increased the number of lipid vacuoles and F4/80+ macrophage (MΦ) and macrophage mannose receptor (MMR)+ M2-MΦs in tumor tissues and adipose tissues surrounding the LN, which was suppressed by BCP. HFD feeding increased the levels of CCL19 and CCL21 in the LN and the expression of CCR7 in the tumor; these changes were blocked by dietary BCP. In vitro culture results revealed that BCP inhibited lipid accumulation in 3T3-L1 preadipocytes; monocyte migration and monocyte chemoattractant protein-1 secretion by B16F10s, adipocytes and M2-MΦs; angiogenesis and lymphangiogenesis. The suppression of adipocyte and M2-cell accumulation and the inhibition of CCL19/21-CCR7 axis may be a part of mechanisms for the BCP suppression of HFD-stimulated melanoma progression.
Introduction

Cutaneous melanoma is one of the most rapidly expanding cancers globally (1). Although curable in its early phases, the survival rates of melanoma wane as the disease advances from the initial site to the sentinel lymph node (LN) and to the distant sites. Sentinel LN biopsy offers prediction of clinical prognosis (reviewed in (2)). Currently, evidence indicates that lymphatic vessel density and lymphatic vessel invasion predict sentinel LN metastasis and lower survival (reviewed in (3)). Therefore, inhibiting lymphatic vessel growth promises to suppress tumor progression.

Tumors are not simply isolated masses of speedily growing tumor cells; the tumor microenvironments are comprised of several dissimilar cell types including monocytes, macrophage (MΦ), fibroblasts, adipocytes, blood endothelial cells and lymphatic endothelial cells (LECs) that join in heterotypic interactions with each other (4). Tumor-associated macrophage (TAMΦs) are one of the important constituents of the tumor microenvironment and may comprise up to 50% of the tumor mass (5). Once educated by the tumor microenvironment, TAMΦs differentiate into alternatively activated M2-MΦs which promote tumor progression by secreting cytokines and pro-angiogenic factors (reviewed in (6)). Additionally, not only tumor cells but also TAMΦs express lymphangiopoietic vascular endothelial growth factor (VEGF)-C and D (reviewed in (7)). Many solid tumors secrete growth factors and cytokines including monocyte chemoattractant protein (MCP)-1 and macrophage-colony stimulating factor (M-CSF) which attract monocytes and stimulate M2 differentiation (8).

Obesity is a risk factor for several cancers (reviewed in (9)) and can lead to worse treatment outcome and prognosis and increase cancer-related mortality (10). The prevalence of obesity has increased dramatically over the past three decades in USA (11) and also in several other parts of the world (12). Results from epidemiological studies indicate that overweight and obesity are associated with increased risk of malignant melanoma (13,14). Recent studies by us (15) and other investigators (16) showed that prolonged high-fat diet (HFD) feeding induced obesity and increased solid tumor growth and LN metastasis in C57BL/6 mice subcutaneously injected with syngeneic B16F10 melanoma cells. Mechanistic associations between cancer and obesity are multidimensional and not well established. Adipose tissue acts as a key endocrine organ by releasing a wide variety of bioactive substances including growth factors and cytokines/chemokines (17). The secretion of bioactive substances is dysregulated in obesity and thus, obesity itself causes subacute, a low grade inflammation (17). This inflammation can provide an environment favorable for tumor promotion and metastasis (reviewed in (18)). Thus, agents with abilities to suppress obesity-induced inflammation can prevent tumors from progressing from a dormant form to a more belligerent and clinically apparent form.

Because adipose tissues are found right beneath the skin, cutaneous melanomas grow in the anatomical neighborhood of adipose tissues that can participate in the heterotypic crosstalk with MΦs, cancer cells, endothelial cells, etc. and stimulate the production of growth factors and cytokines (19). We observed previously that adipocytes were increased in the tumor tissues of HFD-fed mice injected with syngeneic mammary carcinoma cells (20) and B16F10 melanoma cells (15). In addition to tumor tissues, LNs are embedded in adipose tissues, and adipocytes represent one of the most abundant cell types surrounding LNs. These adipocytes surrounding the LN may be increased in obese humans and animals, and their crosstalk with the LN microenvironment can be another stimulus of LN metastasis.

Similar to the tumor microenvironment, the microenvironment within and surrounding the LN can mediate tumor cell metastasis to the sentinel LN as well as the subsequent metastasis to the distant LN and other target organs (reviewed in (21)). Chemotaxis is involved in preferential homing of cancer cells to metastatic sites (22). The chemokine receptor CCR7 are expressed on melanoma cells and their cognate ligands CCL19, and CCL21are secreted abundantly by LECs, thereby mediating LN metastasis of melanoma cells. The overexpression of CCR7 has been shown to stimulate B16F1 melanoma cells into the draining LNs (23). Previously, we reported that HFD feeding to C57BL/6N mice increases the concentrations of CCL19 and CCL21 in the LN and the expression of their receptor CCR7 in B16F10 tumor. We have also shown that the expression of CCL19 and CCL21 in LECs and CCR7 in B16F10s was increased when LECs or B16F10s were co-cultured with mature adipocytes (MAs). These results indicate that HFD-induced increases in concentration gradients of these ligands and receptor expression play a role in the stimulation of LN metastasis of B16F10s (15).

β-Caryophyllene [trans-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo(7.2.0)undec-4-ene, BCP] is a sesquiterpene found in various plants including clove, cinnamon leaves and copaiba balsam (24). BCP was reported to exert anti-oxidant (25) and anti-inflammatory activity (26). Kubo et al. (27) reported that BCP exhibited cytotoxic activity against BT-20 human breast cancer cells, HeLa human cervical cancer cells, HTB140 human melanoma cells and B16F10s. In this study, we demonstrate that dietary BCP suppresses HFD-stimulated solid tumor growth and LN metastasis using the mouse B16F10 tumor model. BCP decreased adipocyte and M2-MΦ accumulation and suppressed CCL19/CCL21-CCR7 signaling axis between the LN and tumor tissues, which may be a part of the mechanisms by/through which BCP suppresses HFD-stimulated tumor promotion.
Materials and methods

Materials

Suppliers of the reagents used in this study were presented in Supplementary Table 1, available at Carcinogenesis Online.

Cell culture

B16F10s were purchased from the Korean Cell Line Bank (Seoul, Korea). B16F10-luc-G5 cells stably expressing firefly luciferase were acquired from Xenogen Corporation (Alameda, CA). These melanoma cells were normally maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with 100 ml/l of fetal bovine serum, 10000 U/l of penicillin and 100 mg/l of streptomycin. Normal adult human lymphatic microvascular endothelial cells (dermal-derived) were purchased from Lonza (Walkersville, MD). These LECs were cultured in endothelial cell basal medium-2 supplemented with EGM-2 SingleQuots (Lonza). The three cell lines were initially expanded and cryopreserved within 1 month of receipt, and all studies with these cells were conducted within five passages of the cryopreserved stocks. The cell suppliers ensure authenticity of these cell lines using short tandem repeat analyses.

To determine the effect of BCP on lymphatic tube formation, LECs were plated at 4 x 10^4 cells/well in Matrigel-coated 24-well plate with various concentration of BCP. After 4 h, photographs were taken for tube formation. M2-Mφs were prepared as described previously (28). Briefly, monocytes were separated from bone marrow cells of the femoral shafts from 20-week-old C57BL/6N mice, and cultured for 7 days with DMEM supplemented with 100 ml/l of heat-inactivated fetal bovine serum. The macrophage monolayers were treated for 16h with 5 μg/l of interleukin-4. MAs were isolated from epididymal fat pads of 20-weeks-old C57BL/6N mice that had been fed the HFD for 16 weeks, as described previously (29). The differentiation of confluent confluent 3T3-L1 preadipocytes was induced as described previously (30). BCP (0, 5 or 10 μmol/l) was present during the 6 day differentiation period. The buildup of cellular lipid droplets was estimated using Oil Red O staining. To determine whether BCP inhibits angiogenesis, aortic ring assay was conducted as described previously (31).

Co-culture

B16F10s, MAs and M2-Mφs were co-cultured using the transwell culture systems (0.4 μm pore size, Corning, Corning, NY). To examine whether BCP inhibits monocyte chemoattractant protein-1 (MCP-1) and M-CSF production, the macrophage monolayers were treated for 16h with 5 μg/l of interleukin-4. MAs were isolated from epididymal fat pads of 20-weeks-old C57BL/6N mice that had been fed the HFD for 16 weeks, as described previously (29). The differentiation of confluent confluent 3T3-L1 preadipocytes was induced as described previously (30). BCP (0, 5 or 10 μmol/l) was present during the 6 day differentiation period. The buildup of cellular lipid droplets was estimated using Oil Red O staining. To determine whether BCP inhibits angiogenesis, aortic ring assay was conducted as described previously (31).

Animals and diets

All experimental protocols were ratified by the Institutional Animal Care and Use Committee of Hallym University (Hallym 2012-2029). Experimental design is provided in Supplementary Figure 1, available at Carcinogenesis Online. Four-weeks-old male C57BL/6N mice (Orient Bio, Gapyung, Korea) were randomly divided into groups (13 mice per group) and fed the following diets: CD (containing 10 kcal% as fat) and HFD (containing 60 kcal% as fat) supplemented with 0.15 or 0.3% of BCP. The CD (No. 12450B) and HFD (No. D12452) were purchased from Research Diets (New Brunswick, NJ), and the supplementation of BCP to HFD and pelleting were performed in Dooyeol Biotech (Seoul, Korea). After 16 weeks of feeding, B16F10-luc-G5 (5 x 10^4 cells suspended in Matrigel) were subcutaneously injected into the right rear flanks of the mice, and all mice were continuously fed the identical diets. Three weeks after injection, the tumor was resected, weighed, divided, and processed for immunofluorescence (IF) staining, real-time RT-PCR analyses, protein arrays, and ELISAs. Mice were killed 2 weeks after the tumor resection, and the weight of the tumors in the draining LN was determined.

Bioluminescence imaging

Tumor cell growth and LN metastasis were monitored by conducting in vivo bioluminescence imaging with an in vivo imaging system-200 (Caliper Life Sciences, Hopkinton, MA). The luciferase substrate D-luciferin (150mg/kg, GoldBio.com, St. Louis, MO) was intraperitoneally injected into mice, and the tumor cell specific bioluminescence (TCSB) was quantified and expressed as photons/s/cm².

IF staining

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, as described previously (20). IF staining was conducted as described previously (28). Fluorochrome-conjugated secondary antibodies (Alexa-488, 594 and 633) were used, and 4’,6-diamidino-2-phenylindole nuclear counterstain was applied. Photographs were obtained using an Axioslmer microscope (Carl Zeiss, Jena, Germany). Staining intensity and the number of immunopositive cells were quantified in five random high-power fields (400x) per section using Image M1 software (Carl Zeiss), and slides were examined in a blinded manner. The results from a mouse (two sections per mouse) were averaged and only one value per mouse (6–8 mice per group) was used for statistical analyses.

Protein array and real-time RT-PCR

To measure the levels of cytokines in tumor tissue lysates, Mouse Cytokine Array Panel A Array Kit and Mouse Angiogenesis Array Kit (R&D Systems) were used (32). For protein arrays, the tissue lysates were pooled (13 animals per group). The expression of proteins was measured using the Image J program [Ver 1.42, NIH Image (Bethesda, MD)], and the values were normalized to those of positive control spots (contained within the membrane).

To validate the array results, the messenger RNA (mRNA) levels of keratinocyte chemokine (KC), MCP-1 and M-SCF of individual tumor samples were estimated by real-time RT-PCR. Total RNA was extracted from tumor tissues, and complementary DNA was synthesized from 1 μg total RNA as described previously (15). Real-time PCR was performed with the indicated primers and cycle profile (Supplementary Table 2, available at Carcinogenesis Online) using Rotor-Gene 3000 PCR (Corbett Research, Mortlake, NSW, Australia). The results were analyzed with Rotor-Gene 6000 Series System Software1.7 program and normalized to those of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Statistical analysis was conducted by using the Statistical Analysis System for Windows, version 9.4 (SAS Institute, Cary, NC), and the R for Windows, version 3.1.1. In SAS, three kinds of procedures [t-test, analysis of variance (ANOVA), NPAR1WAY] were used to test the significance of the differences between the groups in each phenotype. General statistical approaches to adopt for such tests are as follows. First, we performed the Student’s t-test for method testing the differences between the CD group and HFD + 0% BCP group. In order to apply the Student’s t-test, we checked whether the two groups have equal variances or not by conducting a hypothesis testing of equal variance versus unequal variance. If the resulting P value of such a hypothesis test was <0.1, we used the Satterthwaite approximated t-test. If the P value was >0.1, we used the pooled variance t-test. The normality of each phenotype was checked by using its corresponding QQ plot. In case the normality of a certain phenotype was invalidated, a non-parametric test, Wilcoxon rank sum test, was conducted for testing the difference between the two groups by using NPAR1WAY procedure in SAS. However, if more than three samples had equal phenotype, the Student’s t-test was conducted even for the non-normal phenotype.

ANOVA test was performed to determine whether BCP has significant effects on HFD-fed mice. If the ANOVA results indicated differences among the three groups, Duncan’s multiple range tests were conducted to detect which two groups are significantly different. In cases where some phenotypes do not follow the normal distribution, we have conducted the non-parametric test, named Kruskal–Wallis test, by using the NPAR1WAY procedure.
Results

Dietary BCP inhibits lipid accumulation in 3T3-L1 preadipocytes and suppresses body weight gain and fasting blood glucose levels in HFD-fed C57BL/6N mice injected with B16F10s

In vitro culture results with 3T3-L1 preadipocytes revealed that BCP at 5 and 10 μmol/l significantly inhibited lipid droplet accumulation (Supplementary Figure 2, available at Carcinogenesis Online). These concentrations of BCP did not reduce viability of preadipocytes (data not shown). After 21 weeks of feeding, body weights were higher in HFD-fed mice than CD-fed mice. Supplementation of 0.3% BCP in the HFD significantly suppressed the HFD-induced body weight gains. Fasting blood glucose levels were significantly increased in HFD-fed mice, which was prevented by 0.3% dietary BCP. The weights of the spleen and epididymal, mesenteric, retroperitoneal, inguinal and subcutaneous fat were significantly higher in HFD-fed mice than CD-fed mice. Dietary BCP (0.3%) significantly reduced the weights of spleen and mesenteric and subcutaneous fat tissues. The weights of the liver and lung were higher in HFD-fed mice, and these increases were unaffected by dietary BCP (Supplementary Table 3, available at Carcinogenesis Online).

Dietary BCP inhibits HFD-stimulated solid tumor growth in C57BL/6N mice injected with B16F10s

In order to evaluate the effect of BCP on solid tumor growth, B16F10-luc cells were subcutaneously injected into mice that had been fed on experimental diet for 16 weeks and the TCSB, a measure of viable B16F10-luc, was estimated. The TCSB was significantly higher in HFD-fed mice than CD-fed mice 5 days after the injection and remained higher at 12 days. The TCSB was lower in mice fed HFD + 0.3% BCP than that in mice fed HFD + 0% BCP (Figure 1A, Supplementary Figure 3, available at Carcinogenesis Online). The primary solid tumors were resected 21 days after the injection. HFD increased tumor weights, and BCP drastically decreased tumor weights in a dose-dependent manner (P < 0.05) (Figure 1B).

Dietary BCP inhibits HFD-induced cancer cell proliferation and prevents HFD-induced apoptosis reduction in B16F10 tumors of C57BL/6N mice

IF staining results revealed that the expression of Ki67, CDK4, cyclin A and cyclin D1 was markedly higher in the tumors of HFD-fed mice than in CD-fed mice. BCP noticeably suppressed the expression of these cell proliferation-related proteins in tumors in HFD-fed mice (Figure 1C). The number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive apoptotic cells was markedly decreased in the tumors from HFD-fed mice, and 0.3% BCP supplementation in HFD dramatically increased the number of apoptotic cells. The expression of Bcl-2 did not change due to either dietary fat or BCP. The expression of Bax and cleaved (activated) caspase-3 was significantly reduced in the tumor tissues of HFD-fed mice, and these decreases were prevented by dietary BCP (Figure 1D).

Dietary BCP suppresses HFD-induced tumor angiogenesis and lymphangiogenesis

IF staining results showed that the expression of hypoxia-inducible factors 1α (HIF-1α) was increased in the tumor tissues from mice fed on HFD as compared with CD. The expression of HIF-1α substantially decreased in mice fed 0.3% BCP-HFD. The expressions of VEGF-A, CD31, VE-cadherin [a protein expressed only in endothelial cells (34)], and VEGF receptor (VEGFR-2) were substantially increased in the tumors of the HFD-fed mice compared with CD-fed mice. Dietary BCP inhibited HFD-induced increases in the expressions of these proteins (Figure 2C). In agreement with the IF staining results, the results of quantitative RT-PCR analysis also indicated that the mRNA expressions of HIF-1α, VEGF-A, CD31 and VE-cadherin were increased in the tumor tissues of HFD-fed mice relative to the CD-fed mice. Treatment with BCP significantly reduced these transcripts in the tumors of the HFD-fed mice (Figure 2D).

IF staining results revealed that the chronic consumption of HFD markedly increased the expression of VEGF-C, VEGF-D, VEGF-R3 and lymphatic vessel endothelial receptor (LYVE)-1 in the tumor tissues. The HFD-stimulated expression of VEGF-C, VEGF-D, VEGF-R3 and LYVE-1 was prevented by BCP supplementation (Figure 2C). The results of quantitative RT-PCR analysis showed that HFD feeding drastically increased the mRNA expression of VEGF-C, VEGFR-3 and LYVE-1 in tumor tissues, which was significantly suppressed by 0.3% BCP supplementation (Figure 2D). In vitro culture results revealed that BCP-suppressed microvessel outgrowth from the mouse aorta (Figure 2A) and capillary-like tube formation of LECs (Figure 2B).

Dietary BCP prevents HFD-induced accumulation of adipocytokines and M2-MΦs and increases in the levels of growth factors/cytokines in tumor tissues

The results of Oil red O staining showed that chronic HFD feeding increased the lipid accumulation in tumor tissues, and this increase was hampered by BCP treatment (Figure 3A and B). The number of CD45+ leukocytes, F4/80+ MΦs, and macrophage mannose receptor (MMR)-M2-MΦ cells was higher in the tumor of HFD-fed mice than CD-fed mice. BCP supplementation markedly suppressed the HFD-induced increases in leukocytes and macrophages in tumor tissues (Figure 3A). Additionally, the mRNA expressions of F4/80 and MMR in tumor tissues were increased by HFD feeding, which were significantly suppressed by 0.15% BCP. By contrast, the administration of HFD or BCP had no effect on the mRNA expression of CD11c, an M1-MΦ marker (Figure 3C).

Protein array results revealed that the levels of CXCL9, M-CSF, VEGF-A, KC, MMP-3, MCP-1, etc. were increased in the tumor of HFD-fed mice, which was suppressed by dietary BCP (Supplementary Table 4, available at Carcinogenesis Online). Consistent with the array results, the mRNA levels of KC, M-CSF and MCP-1 were markedly increased in the tumor tissue of HFD-fed mice, which was suppressed by dietary BCP (Figure 3D).

BCP suppresses MCP-1 secretion by B16F10s, MΦs and M2-MΦs and monocyte migration induced by CM of these cells

To examine whether BCP directly inhibits MCP-1 and M-CSF secretion by these cells, the concentration of these cytokines in
CM was assessed by ELISAs. BCP (5 μmol/l) decreased the concentration of MCP-1 in the CM of B16F10s, M2-Mφs and MAs and that of M-CSF in M2-Mφ CM only. The concentrations of MCP-1 and M-CSF were increased in co-culture CMs of B16F10s, MAs and M2-Mφs, and these increases were suppressed by BCP treatment (Figure 4A). The concentration of BCP did not reduce the viability of B16F10 cells, LECs, monocytes or M2-Mφs (Supplementary Figure 4, available at Carcinogenesis Online). Monocyte migration was moderately increased when B16F10 or MA CM was used as a chemoattractant, and BCP (5 μmol/l) significantly inhibited the CM-induced monocyte migration. The CM of MA/B16F10/M2-Mφ co-culture further stimulated monocyte migration, which was inhibited by 5 μmol/l BCP (Figure 4B).

Dietary BCP suppresses HFD-induced LN metastasis of B16F10s in C57BL/6N mice

Metastasis into the draining LN was measured on 12 days after the tumor resection by quantifying TCSB. The TCSB in the LN was markedly increased in HFD-fed mice as compared with CD, which was prevented by 0.3% dietary BCP (Figure 5A). When animals were killed at 14 days after the resection, the weights of tumor nodules in the draining LN were tremendously higher in HFD-fed mice than CD-fed mice, but this increase was blocked by 0.3% BCP in the diet (Figure 5B). The size of the LN was substantially increased in HFD-fed mice (Figure 5C) as well as the number of Mφs, especially MMR-Mφs in adipose tissues surrounding the LN, and these...
changes were suppressed by dietary BCP ([Figure 5D](#fig5d)) and ([Figure 5E](#fig5e)).

Dietary BCP suppresses HFD-induced increases in the concentrations of CCL19 and CCL21 in the LN as well as the expression of CCR7 in tumor cells

ELISA results revealed that the concentrations of CCL19 and CCL21 were significantly higher in the LN than those in the tumor. HFD feeding significantly increased the levels of CCL19 and CCL21 in the LN, and these increases were blocked by BCP ([Figure 6A](#fig6a)). There was a slight increase in CCL19 in tumor tissues of HFD-fed mice, and this increase was prevented by dietary BCP. The mRNA expression of CCL19 and CCL21 in the LNs was significantly increased in HFD-fed mice, and these increases were blocked by dietary BCP ([Figure 6B](#fig6b)). IF staining and quantitative RT-PCR results revealed that HFD feeding markedly increased CCR7 expression in the tumor tissues, which was prevented by dietary BCP ([Figure 6C](#fig6c)). Cell culture results showed that BCP (5 μmol/l) reduced CCR7 mRNA expression in B16F10s. CCR7 transcripts were markedly elevated in B16F10s when co-cultured with MAs, and these increases were suppressed by 5 μmol/l BCP ([Figure 6D](#fig6d)).

Subcutaneous adipose tissue mass (SATM) is strongly correlated with the expression of cytokines/chemokines

In order to assess the strength of the association between SATM and the markers altered in tumor-bearing mice, we...
calculated Pearson correlation coefficients ($r$). Strong correlations were observed between the body weight and SATM ($r = 0.967$); between SATM and tumor MCP-1 mRNA ($r = 0.803$); between SATM and tumor KC mRNA ($r = 0.783$); between SATM and tumor M-CSF mRNA ($r = 0.831$); between SATM and tumor CCR7 mRNA ($r = 0.904$); between SATM and LN CCL19 protein ($r = 0.633$); and between SATM and LN CCL21 protein ($r = 0.687$) (Supplementary Figure 5, available at Carcinogenesis Online).

**Discussion**

The present study demonstrate that dietary BCP suppresses HFD-induced (i) body weight gain; (ii) solid tumor growth and LN metastasis; (iii) angiogenesis, lymphangiogenesis, cell proliferation and cell survival in tumor tissues; (iv) accumulation of adipocytes and M2-MΦs as wells as increases in the levels of cytokines including KC, MCP-1 and M-CSF in tumor tissues; (v) increases in the concentrations of CCL19 and CCL21 in the LN and the expression of CCR7 in tumor tissues; (vi) increases in M2-MΦs in the adipose tissues surrounding LN. Additionally, in vitro cell culture studies demonstrate that BCP directly suppresses (i) lipid accumulation in 3T3-L1 preadipocytes; (ii) microvessel outgrowth from the mouse aorta and capillary-like tube formation of LECs; (iii) MCP-1 production by B16F10s, M2-MΦs and adipocytes and M-CSF production by M2-MΦ; (iv) monocyte migration induced by B16F10s, M2-MΦs and adipocytes. These results indicate that the suppression of adipocyte and MΦ accumulation by dietary BCP is an important mechanism for suppression of tumor growth and metastasis. However, BCP exerts direct effects on monocyte migration, angiogenesis and lymphangiogenesis.

![Figure 3. BCP suppresses HFD-stimulated expression of cytokines and accumulation of lipid droplets and M2 macrophages in B16F10 solid tumors. (A) Tumor sections were stained with Oil red O and antibody raised against CD45, F4/80, CD11c or MMR. Representative images of the staining are shown. Scale bar, 50 μm. (B) Lipid vacuoles were counted ($n = 5$). (C, D) Total RNA was isolated, reverse transcribed, and real-time PCR was conducted ($n = 7$). Each bar represents the mean ± SEM. *Significantly different from the CD group, $P < 0.05$. Means without a common letter differ among the three HFD groups, $P < 0.05$.](https://academic.oup.com/carcin/article-abstract/36/9/1028/474827)
BCP is commonly ingested with food containing vegetables, especially with spices and essential oil. A fair amount of BCP can be found in food spices including oregano, cinnamon, cloves, rosemary and black pepper. Gertsch et al. (35) estimated the daily intake of this lipophilic sesquiterpene to be around 10–200 mg, which could potentially modulate inflammatory responses and other pathophysiological processes via the regulation of cannabinoid receptor type 2 activation. Our results indicate that BCP at the concentration of 1.5 g/kg of diet exerts preventive effects on cancer progression in mice through the inhibition of obesity-associated inflammation. Taken together, these results indicate that increasing the intake of spices such as cloves, oregano, cinnamon and black pepper would help to prevent melanoma growth and metastasis. A previous study reported that BCP oral doses of up to 5 g/kg body weight revealed no signs of toxicity in mice (36), suggesting that BCP can also be taken as an oral supplement independently from the diet. However, it is difficult to extrapolate the effective animal doses to human equivalent doses. Thus, future studies are needed to determine whether and/or how much BCP should be recommended as an oral supplement independently from the diet for people who have a high risk of melanoma with HFD consumption.

A previous study reported that oral doses of BCP up to 5 g/kg body weight revealed no sign of toxicity in mice (36). In the present study, the concentrations of BCP (0.15 and 0.3%) in the diet were chosen because of the report by Cho et al. (26), that oral administration of BCP at 300 mg/kg/day significantly reduced inflammation of the colon in mice exposed to dextran sulfate sodium. If a mouse weighing 30 g consumes 3 g/day of diet containing 0.3% BCP, this would make the daily intake 300 mg/kg body weight. However, there is a paucity of information on the oral bioavailability of this compound. As this compound was found to be strongly absorbed through cell membranes (37), the bioavailability of ingested BCP is expected to be high. A previous study reported that the plasma concentration was 0.12 μg/ml (0.6 μmol/l) at 3.5 h after a single oral dose of 50 mg/kg BCP, whereas the half-time of the compound was 4.07 h. It was assumed that the blood concentrations (approximately 2.5–5 μmol/l) of BCP could be achievable when mice were fed the 0.3% BCP diet. However, we did not determine the concentrations of BCP in the blood from these mice. Future study is also needed to examine how much BCP is needed to achieve these concentrations in the blood of humans, and whether or not such doses are toxic.

In the present study, dietary BCP suppressed HFD-induced increases in cell proliferation and survival in tumor tissues (Figure 1C and D). In vitro results revealed that 10 μmol/l BCP significantly decreased B16F10 viability (Supplementary Figure 4A, available at Carcinogenesis Online). These results are consistent with those of the earlier report that BCP exerts cytotoxic effect against B16F10s (IC50 = 3.88 μg/ml; 19 μmol/l) (27). Taken together, the present results indicate that the inhibition of cell proliferation and the induction of apoptosis resulting from BCP administration are a part of the mechanisms by which BCP inhibits solid tumor growth.

Angiogenesis performs a critical role in the growth and metastasis of most tumors, including melanomas (38). It appears that healthy persons can hold tiny tumors and dysplastic foci for a long time without symptoms or detection. These lesions do not expand in the absence of angiogenesis or inflammation (reviewed in (39)). New blood vessels in tumor tissues provide oxygen, nutrients and growth factors for the highly proliferating
malignant cells to flourish, while allowing tumor cells to get into the circulation. For this reason, the inhibition of angiogenesis is considered to be an attractive strategy for cancer prevention. In human malignant melanoma, the numbers of Mφs and microvessels were shown to increase with increasing depth of tumor and tumor angiogenesis (40). In the present study, F4/80+ TAMPφs were increased in HFD-fed mice, which was prevented by dietary BCP (Figure 3A and C). Mφs may promote angiogenesis by secreting proangiogenic growth factors and proteases including VEGF-A, matrix metalloproteinase-9 and urokinase-type plasminogen activator (41). VEGF-A activates its specific receptor VEGF-R2, resulting in promotion of angiogenesis (42). The current study revealed that dietary BCP suppressed HFD-induced increases in CD31, VE-cadherin, VEGF-A and VEGF-R2 expression (Figure 2C and D). Taken together, these results indicate that BCP decreases Mφ infiltration, which helps to suppress the VEGF-A/VEGF-R2 axis, thereby inhibiting tumor angiogenesis in HFD-fed mice. This reduced angiogenesis may be one of the important reasons for reduced tumor growth in BCP-fed mice.

Tumor-associated lymphangiogenesis enhances LN metastasis in experimental cancer models and is a prognostic indicator for metastasis and overall patient survival (34–46). The activation of VEGF-R3 pathway via binding to the specific lymphangiogenesis factors VEGF-C and VEGF-D promotes tumor lymphangiogenesis and LN metastasis (44,45). Additionally, blockade of the VEGF-R3 pathway inhibits tumor
lymphangiogenesis and LN metastasis in various experimental tumor models, suggesting that the VEGF-R3 pathway may represent a target for the prevention of human cancer (reviewed in (47)). In the present study, we noted that dietary BCP drastically suppressed HFD-promoted LN metastasis (Figure 5A) as well as HFD-induced increases in the protein and mRNA expressions of LYVE-1, VEGF-C, VEGF-D and VEGF-R3 (Figure 2C and D). Taken together, these findings indicate that BCP suppression of HFD-stimulated LN metastasis is probably attributed, at least in part, to the decreased lymphangiogenesis resulting from a reduction in the expression of VEGF-C, VEGF-D and VEGF-R3.

In solid tumors, hypoxia is widespread since the newly made blood vessels in tumors are easy to collapse and frequently chaotic; it is also because cancer cell proliferation overpowers angiogenesis. In response to intratumoral hypoxia, HIF-1α is activated and then plays a vital role in the adaptation of tumor cells to hypoxia as a transcription factor of wide array of genes implicated in tumor angiogenesis as well as the proliferation, survival, invasion and metastasis of tumor cells (48). Overexpression of HIF-1α is shown to be a marker of highly belligerent disease and associated with treatment failure and poor prognosis in several cancers (49,50). Olsson et al. (51) reported that HIF-1α-dependent VEGF-A

Figure 6. BCP suppresses HFD-stimulated secretion of CCL19 and CCL21 in the LN and the expression of CCR7 in the tumor. (A) Mice were fed, injected with B16F10-luc cells, and then killed as described in Figure 4. (A) LN and tumor tissue lysates were prepared for the determination of CCL19 and CCL21 levels by the respective ELISA kits (n = 5). (B) Total RNA was isolated, reverse transcribed, and real-time PCR was conducted (n = 7). (C) Tumor sections were stained with an antibody raised against CCR7. Representative images of the IF staining are shown. Scale bar, 50 μm. (left panel). Real-time RT-PCR was conducted with tumor lysates (right panel) (n = 5). (A–C) Each bar represents the mean ± SEM. *Significantly different from the CD group, P < 0.05. Means without a common letter differ among the three HFD groups, P < 0.05. (D) B16F10s, M2-MΦ and MAs were co-cultured and treated with BCP as described in Figure 4. Total RNA was isolated from B16F10 cells and real-time RT-PCR was performed. #Significantly different from the B16F10 (0 μmol/l BCP) only culture, P < 0.05. *Significantly different from the respective 0 μmol/l BCP treatment cultures, P < 0.05.
expression strongly correlated with poor cancer prognosis. Recent studies have revealed that HIF-1α correlates with VEGF-C expression and lymphangiogenesis (52). In the present study, we observed that BCP inhibited HFD-stimulated expression of HIF-1α in the tumors as well as that of VEGF-A and VEGF-C. Additionally, increased VEGF-A in the hypoxic region can act as a chemoattractant for monocytes and macrophages and can, therefore, play an important role in the recruitment of MΦs into the hypoxic region (reviewed in (53)). MΦs secrete proangiogenic growth factors and proteases (41) as well as lymphangiogenic factors (7). Taken together, these results indicate that dietary BCP decreases HIF-1α expression and the subsequent expression of VEGF-A and VEGF-C, thereby suppressing tumor angiogenesis and lymphangiogenesis.

Tumor cells and adipocytes generate various chemokines, such as MCP-1 and M-CSF which promote MΦ recruitment and the differentiation toward M2-MΦs (8). We have reported previously that the HFD feeding stimulates the accumulation of lipids and M2-MΦs in B16F10 tumors and adipose tissues in C57BL/6N mice and increased the secretion of various cytokines including M-CSF and MCP-1 (15). We have also shown that adipocytes produce chemoattractants for monocyte infiltration and the cross talk between B16F10s, adipocytes and M2-MΦs produces substances that stimulate lymphangiogenesis and tumor cell migration. The crosstalk between M2-MΦs and tumor cells stimulates VEGF-C expression in M2-MΦs and VEGF-A expression in tumor cells. Additionally, adipocytes stimulate VEGF-R3 expression in LECs and VEGF-D in M2-MΦs. In the present study, HFD-induced accumulation of lipids and M2-MΦs in tumor tissues and adipose tissues surrounding the LN were significantly suppressed by dietary BCP (Figures 3A–C and 5D–F). Taken together, these results suggest that BCP inhibits adipocyte accumulation in HFD-fed mice, which leads to changes in tumor and LN microenvironments (decreases in MΦ accumulation and cytokine/chemokine production), leading to the inhibition of tumor promotion.

In vitro cell culture studies revealed that BCP directly inhibits the production of MCP-1 in B16F10s, M2-MΦs and MAs, and that of M-CSF in M2-MΦs. The concentrations of MCP-1 and M-CSF were increased in CM of co-cultures with B16F10s, MAs and M2-MΦs, and these increases were suppressed by BCP treatment (Figure 4A). BCP inhibits monocyte migration induced by B16F10 or MA CM and also migration by the CM of MA/B16F10/M2-MΦ co-culture (Figure 4B). These results indicate that BCP directly inhibits chemokine production in adipocytes, tumor cells and M2-MΦs, in addition to exerting indirect inhibitory effects via decreasing adipocyte and M2-MΦ accumulation in tumor tissues.

Using the same mouse tumor models, we have shown previously that HFD feeding increases the concentration gradients of CCL19 and CCL21 between the tumor and LN and the expression of their receptor CCR7 in the B16F10 tumor (15). The present results revealed that BCP suppresses these HFD-induced changes in CCL19/21–CCR7 axis (Figure 6A–C). In vitro cell culture results revealed that BCP directly inhibited CCR7 mRNA expression in B16F10 cells as well as MA-induced increases in CCR7 mRNA expression (Figure 6D). These results indicate that BCP directly suppresses CCR7 expression in B16F10s along with exerting indirect inhibitory effects by decreasing adipocyte accumulation in tumor tissues.

Adipose tissue acts as an important endocrine organ via the synthesis of several adipokines which are involved in the regulation of insulin sensitivity, lipolysis, control of energy intake and inflammatory processes. The adipocyte is the center of the development of obesity-induced inflammation, induced by increasing the secretion of various pro-inflammatory chemokines and cytokines (reviewed in (17)). Cutaneous melanomas grow in the anatomical neighborhood of subcutaneous adipose tissues, and the draining LN are imbedded in subcutaneous fat. In the present study, BCP reduced the HFD-induced increases in SATM. (Supplementary Table 3, available at Carcinogenesis Online). Additionally, strong positive relationships were observed between the SATM and the mRNA expressions of MCP-1, M-CSF, KC and CCR7 in tumor tissues, as well as the protein expression of CCL19 and CCL21 in the LN (Supplementary Figure 5, available at Carcinogenesis Online). Taken together, these results suggest that some effects of dietary BCP on melanoma progression may be indirect, resulting from BCP-induced changes in body weight or subcutaneous fat.

In conclusion, we demonstrated that dietary BCP suppressed body weight gain, solid tumor growth and LN metastasis in HFD-fed C57BL/6N mice harboring B16F10 melanoma allografts. These inhibitory effects of BCP were associated with the suppression of cell cycle progression, cell survival, angiogenesis and lymphangiogenesis in the tumors. Dietary BCP also suppressed HFD-induced accumulation of lipids and M2-MΦs in the tumor and adipose tissues surrounding the LN as well as HFD-induced increases in cytokine secretion. Dietary BCP reduced HFD-induced increases in CCL19 and CCL21 levels in the LN and CCR7 expression in tumor. These results indicate that the inhibition of lipid accumulation, thereby suppressing obesity-associated changes in tumor and LN microenvironments, is one of the mechanisms by/through which BCP inhibits solid tumor growth and LN metastasis. Additionally, BCP exhibited direct inhibitory effects on monocyte migration; the secretion of MCP-1 in B16F10s, M2-MΦs and MAs and that of M-CSF in M2-MΦs; and CCR7 mRNA expression in B16F10s. These findings suggest that BCP can be recommended to individuals who have a risk of melanoma and are consuming a HFD.

Supplementary material
Supplementary Figures 1–5 and Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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References
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