Blackberry Extracts Inhibit Activating Protein 1 Activation and Cell Transformation by Perturbing the Mitogenic Signaling Pathway

Rentian Feng, Linda L. Bowman, Yongju Lu, Stephen S. Leonard, Xianglin Shi, Bing-Hua Jiang, Vince Castranova, Val Vallyathan, and Min Ding

Abstract: Blackberries are natural rich sources of bioflavonoids and phenolic compounds that are commonly known as potential chemopreventive agents. Here, we investigated the effects of fresh blackberry extracts on proliferation of cancer cells and neoplastic transformation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), as well as the underlying mechanisms of signal transduction pathways. Using electron spin resonance, we found that blackberry extract is an effective scavenger of free radicals, including hydroxyl and superoxide radicals. Blackberry extract inhibited the proliferation of a human lung cancer cell line, A549. Pretreatment of A549 cells with blackberry extract resulted in an inhibition of 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation induced by ultraviolet B (UVB) irradiation. Blackberry extract decreased TPA-induced neoplastic transformation of JB6 P+ cells. Pretreatment of JB6 cells with blackberry extract resulted in the inhibition of both UVB- and TPA-induced AP-1 transactivation. Furthermore, blackberry extract also blocked UVB- or TPA-induced phosphorylation of ERKs and JNKs, but not p38 kinase. Overall, these results indicated that an extract from fresh blackberry may inhibit tumor promoter-induced carcinogenesis and associated cell signaling, and suggest that the chemopreventive effects of fresh blackberry may be through its antioxidant properties by blocking reactive oxygen species-mediated AP-1 and mitogen-activated protein kinase activation.

Introduction

Previous studies have demonstrated the chemopreventive effects of fruits and vegetables in animals and humans (1–3). Epidemiological investigations suggest that diet composition plays an important role in cancer risk control (4,5). Among the potential chemoprotective diets, growing attention has been dedicated to berry products, such as blackberry, strawberry, cranberry, black raspberry, and blueberry (6–10). Although these berry products have been reported to possess anti-inflammatory and chemopreventive effects (6,9,10–14), little is known about the underlying mechanisms at the cellular and molecular levels.

The AP-1 signal transduction pathway is known to be an important molecular target of chemopreventive strategies. AP-1, a ubiquitous transcriptional activator, is composed of members of the Jun and Fos families that form homodimers or heterodimers and bind to a distinct DNA response element. Elevated AP-1 activities and its upstream regulators, mitogen-activated protein kinases (MAPK), are involved in many disease processes, such as inflammation, neoplastic transformation, tumor progression, metastasis, and angiogenesis (15–19). Reactive oxygen species (ROS) produced by cells exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA) or ultraviolet (UV) irradiation can result in rapid activation of MAPKs, ERKs, JNKs, and p38 (20). The development of an oxidant/antioxidant imbalance in preneoplasia may activate redox-sensitive transcription factor AP-1, which controls the expression of various genes implicated in inflammatory processes, cell differentiation, and stress responses (19,21,22). Blockade of TPA-induced AP-1 activation has been shown to inhibit neoplastic transformation (23). Inhibition of AP-1 activity in transformed JB6 RT101 cells causes reversion of the tumor phenotype (24). Furthermore, the role of AP-1 transactivation in tumor promotion has been demonstrated using transgenic mice (25). Therefore, elevated AP-1 activity has been implicated as causal in transformation responses to tumor promoters. A potential chemopreventive strategy would be to block MAPK-AP-1 signal activation induced by environmental carcinogens using natural antioxidants.

Evidence for the involvement of oxidative stress in tumor promotion has accumulated in the past years. The antioxidant theory of cancer prevention has been widely documented (15). Early studies reported that natural antioxidants from vegetables or fruits could inhibit cancer cell growth and induce apoptosis (26–28). It has been proposed that the consumption of whole fruits may provide the antioxidant bal-
ance needed to quench ROS, which has been implicated in tumorigenesis (29). To elucidate the mechanism of the anticarcinogenesis effects of phytochemicals, we investigated the effect of a blackberry extract on ultraviolet B (UVB) or TPA-induced AP-1-MAPK activation and neoplastic transformation. The effect of fresh blackberry extract on the formation of 8-hydroxy-2’-deoxyguanosine (8-OHdG), an important biomarker of oxidative DNA damage (30), induced by UVB was also examined.

**Materials and Methods**

**Cell Lines and Reagents**

The JB6 P+ mouse epidermal cell line, which was or was not stably transfected with the AP-1-luciferase reporter plasmid (JB6/AP/kB; 31), was cultured in Eagle’s MEM (EMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin). Human lung cancer cell line A549 was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS. The cells were cultured at 37°C in a 5% CO₂ atmosphere.

EMEM and DMEM were obtained from Whittaker Biosciences (Walkersville, MD). FBS, L-glutamine, and penicillin and streptomycin were purchased from Life Technologies, Inc. (Gaithersburg, MD). Luciferase assay kit was obtained from Promega (Madison, WI). PhosphoPlus MAPK antibody kits were purchased from New England BioLabs (Beverly, MA). Monoclonal antibody against 8-OHdG was purchased from Genox (Baltimore, MD). The Vectastain-ABC kit was from Vector Laboratories (Burlingame, CA).

Fresh blackberry was obtained from Kroger, Inc., in the harvest season. The fruits were washed with a mild detergent and rinsed three times with Milli-Q deionized water to remove possible pesticide and preservative residues. Blackberry extract was prepared using the protocol as described previously (32). Briefly, 1 ml of distilled water per gram of washed blackberry was mixed and blended at high speed. The blended homogenate was strained, centrifuged, and filter sterilized and stored at −20°C. The protein contents in filtrates were determined by PIERCE Protein Assay Reagents (Rockford, IL). The blackberry extracts used in this study were standardized based on the protein contents to a normalized value.

**Free Radical Measurements**

The scavenging efficiency of OH or O₂⁻ radicals by blackberry extracts was determined using an electron spin resonance (ESR) spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly, as described previously (33). The spin trap, DMPO, was purified by charcoal decolorization and vacuum distillation. The DMPO solution, thus purified, did not contain any ESR detectable impurities. The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants. Reactants were mixed in test tubes in a final volume of 1.0 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. Experiments were performed at room temperature and under ambient air.

**Determination of 8-OHdG**

Formation of 8-OHdG induced by UVB irradiation was measured by immunocytochemistry in A549 cells. Cells were seeded on four-chamber slides (Falcon) at a concentration of 50,000 cells per chamber and incubated at 37°C for 48 h. Cells were pretreated with or without blackberry extract for another 1.5 h and then exposed to UVB irradiation (6.3 kJ/m²). Cells were washed with phosphate buffered saline (PBS) and fixed in cold acetone. Immunocytochemistry was performed as described by Knaapen et al. (35). The Vectastain-ABC kit was used for immunohistochemical staining according to the manufacturer’s protocol. The 8-OHdG was quantified using digital imaging analysis system (SimplePCI, Compix Inc., Cranberry Township, PA). The total staining intensity of each slide was measured and divided by the number of cells evaluated. A minimum of 300 cells were evaluated per sample using three pictures.

**Cell Growth Measurement by Electric Cell-Substrate Impedance Sensor (ECIS)**

ECIS (Model 1600R, Applied BioPhysics, Troy, NY) assay, a widely used technique for measurement of cell attachment, spreading, and proliferation (36–38), was employed to monitor the growth of cultured cells. Cells (1 × 10⁴) suspended in 400 µl of medium with or without blackberry extract were seeded on electrodes. The electrodes were pre-coated with the same medium for 0.5 h before using. The cells were equilibrated in the incubator for 15 min. A constant current source applied an AC signal of 1 µA at 4 kHz between a small active electrode (250 µm diameter) and a large counter electrode to complete the circuit. The rate of cell proliferation on the microelectrode was monitored for 72 h as real-time changes in resistance.

**Assay of AP-1 Activity in Vitro**

A confluent monolayer of JB6/AP/kB cells was trypsinized, and 5 × 10⁴ viable cells (suspended in 1 ml of EMEM supplemented with 5% FBS) were added to each well of a 24-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. Twelve hours later, cells were cultured in EMEM supplemented with 0.5% FBS for 24 h to minimize basal AP-1 activity. The cells were pretreated with...
or without blackberry extract at the concentrations indicated for 1 h and then exposed to TPA (20 ng/ml) for 48 h or UVB (4 kJ/m²) irradiation followed by incubation at 37°C for additional 16 h. The cells were extracted with 200 µl of 1 × lysis buffer provided in the luciferase assay kit by the manufacturer (Promega, Madison, WI). Luciferase activity was measured using a Monolight Luminometer, Model 3010 (32). The results were expressed as relative AP-1 activity, which was normalized to the untreated controls.

Protein Kinase Phosphorylation Assay

Immunoblots for phosphorylation of ERKs, JNKs, and p38 kinase were carried out using PhosphoPlus MAPK antibody kits as described in the protocol of New England BioLabs. Phosphospecific antibodies were used to detect phosphorylated sites of ERKs, JNKs, and p38 kinase (39). Nonphosphospecific antibodies against ERKs, JNKs, and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay by using the same transferred membrane blot.

Anchorage-Independent Transformation Assay

For soft agar assays, JB6 P+ cells (1 × 10⁴) were exposed to TPA (20 ng/ml) in the presence or absence of blackberry extract, in 0.33% Bacto-agar containing 20% FBS over 0.5% agar medium containing 15% FBS EMEM medium. The cultures were maintained in a 37°C, 5% CO₂ incubator for 2 wk and the colonies were counted as described by Colburn et al. (40).

Statistical Analysis

Data presented are the means ± standard errors of values compared and analyzed using one-way analysis of variance test (SigmaStat statistical software, Jandel Scientific, CA) to assess the statistical significance between treatments. Statistical significance was set at P < 0.05.

Results

Scavenging of Free Radicals by Fresh Blackberry Extract

To study the potential antioxidant activity of blackberry, ESR was utilized to measure the ability of blackberry extract to scavenge OH and O₂⁻ radicals. The Fenton reaction (Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH⁻) was used as a source of OH radicals. As shown in Fig. 1A, a typical spectrum of OH radicals was generated by a 1:2:2:1 quartet indicating the DMPO/OH adduct. The addition of blackberry extract normalized as protein concentration reduced the DMPO/OH spectra in a concentration-dependent manner, demonstrating the ability of blackberry extract to scavenge the OH radical (Figs. 1B and 1C).

Superoxide radicals were generated using a xanthine/xanthine oxidase system and measured using ESR. Figure 2A shows the spin adduct spectrum generated from xanthine and xanthine oxidase in the presence of DMPO. Analysis of the spectrum shows hyperfine splittings at a_N = 14.2 G, a_H = 11.5 G, and a_H¹ = 1.2 G. These splittings are typical of the DMPO/O₂⁻ spectra. The addition of blackberry extract normalized as protein concentration reduced the DMPO/O₂⁻ adduct signal, demonstrating the ability of blackberry extract to scavenge the O₂⁻ radical (Figs. 2B and 2C).

Blackberry Extract Suppresses Cancer Cell Proliferation

The inhibitory effect of blackberry extract on the proliferation of cancer cell line A549 was examined by ECIS assay. As shown in Fig. 3, a concentration-dependent reduction in resistance was observed in A549 cells treated with blackberry extract, indicating cell proliferation was inhibited by blackberry extract (Fig. 3A). Microscopic analysis indicated
that cell spreading was significantly inhibited in blackberry extract-treated cells (data not shown).

To confirm the inhibition of A549 cell growth by fresh blackberry extract, the cells were trypsinized, and the cell number in each well was counted at the end of experiments, using Coulter® Multisizer II Cell Counter (Coulter Electronics Ltd., Luton, UK). After treatment of A549 cells with blackberry extract (120 µg protein/ml), the number of cells decreased by 62.6% (P < 0.05; Fig. 3B). However, no significant inhibition was observed in JB6 cells treated with blackberry extract within the same dose range by ECIS assay in the experiment (data not shown).

**Blackberry Extract Decreases UVB-Induced 8-OHdG Formation**

DNA oxidative damage is a critical event for the initiation of mutagenic lesion. Formation of 8-OHdG has been shown to be an important biomarker of DNA oxidative damage (30). We investigated the effects of blackberry extract against UVB-induced 8-OHdG formation using immunocytochemistry.

As shown in Figs. 4A and 4B, 8-OHdG formation as evidenced by the staining intensity in UVB-exposed A549 cells increased 32% compared with untreated control cells. However, with pretreatment of cells with 0.6 mg protein/ml blackberry extract, the formation of 8-OHdG decreased 16% compared with UVB-treated positive control cells, demonstrating that blackberry extract could prevent UVB-induced DNA damage.

**Inhibition of AP-1 Activity by Blackberry Extract**

ROS is involved in AP-1 activation, and AP-1 plays an important role in carcinogenesis induced by tumor promot-
ers (17,25). Thus we investigated the effects of blackberry extract on AP-1 activation. Pretreatment of JB6 cells with blackberry extract produced a dose-dependent inhibition on AP-1 activity induced by either UVB or TPA (Figs. 5 and 6). The AP-1 activity induced by TPA or UVB was inhibited by 36–100% or 5–78% over the tested dose range of 30, 60, or 120 µg/ml, respectively (Figs. 5 and 6). Blackberry alone did not show any influence on basal AP-1 activation (Figs. 5 and 6).

Effect of Blackberry Extract on TPA-induced Cell Transformation

To study whether blackberry extract has inhibitory activities for TPA-induced neoplastic transformation, anchor-age-independent transformation of JB6 P+ cells was determined in the presence or absence of blackberry extract. As shown in Fig. 7, TPA efficiently induced JB6 P+ cell transformation after 2 wk of culture. The addition of blackberry extract significantly suppressed TPA-induced cell transformation in a concentration-dependent manner (P < 0.05). Blackberry extract alone had no effect on spontaneous cell transformation (Fig. 7).

Effects of Blackberry Extract on TPA- or UVB-Induced MAPKs Activation

AP-1 has been identified as a target of the MAPK family, including ERKs, JNKs, and p38 kinase (15,17,41). Because MAPKs are the upstream kinases responsible for c-Jun phosphorylation and AP-1 activation, we next tested which class of MAPK is involved in the inhibition of AP-1 activation by blackberry extract. We examined the influences of blackberry extract on TPA- or UVB-induced phosphorylation of ERK1/2, JNKs, and p38 kinase in JB6 P+ cells. Pretreatment of cells with blackberry extract led to a substantial inhibition of phosphorylation of ERKs and JNKs induced by TPA or UVB in a dose-dependent manner, but not p38 kinase (Figs. 8 and 9). These results indicate that the inhibition of AP-1 activation by blackberry may be mediated through the inhibition of ERKs or JNKs signaling in JB6 cells.

To test the effect of ERKs activation on TPA-induced cell transformation and AP-1 activation, PD98059 was utilized. PD98059 is a well-known specific MEK/ERK signal inhibitor. As expected, preincubation of JB6/AP/kB cells with PD98059 significantly suppressed both TPA- and UVB-induced AP-1 activation (Figs. 10A and 10B), as well as TPA-induced neoplastic transformation in JB6 P+ cells (Fig. 10).
Figure 5. Blackberry extract suppresses TPA-induced AP-1 activity. JB6/AP1κB cells that stably transfected with AP-1 luciferase reporter plasmid were cultured as described in Materials and Methods. The cells were pretreated with or without blackberry extract at the concentrations indicated for 1 h and then were exposed to TPA (20 ng/ml) and cultured for an additional 48 h. AP-1 activity was determined by luciferase assay. Results, presented as relative AP-1 induction compared with untreated control cells, are expressed as means and standard errors from three wells. The experiment was repeated three times. *Indicates a significant inhibition of TPA-induced AP-1 activation by blackberry extract (P < 0.05).

Figure 6. Blackberry extract suppresses UVB-induced AP-1 activity. JB6/AP1κB cells were cultured as described in Materials and Methods. The cells were pretreated with or without blackberry extract at the concentrations indicated for 1 h and then were exposed to UVB irradiation (4 kJ/m²) and cultured for an additional 16 h. AP-1 activity was determined by luciferase assay. Results, presented as relative AP-1 induction compared with untreated control cells, are expressed as means and standard errors from three wells. The experiment was repeated three times. *Indicates a significant inhibition of UVB-induced AP-1 activation by blackberry extract (P < 0.05).

Figure 7. Inhibition of TPA-induced cell transformation by blackberry extract. JB6 P+ cells (1 x 10⁴) were exposed to TPA (20 ng/ml) in the presence or absence of blackberry extract in 0.33% agar for 14 days and scored for colonies at the end of the experiments. Results, presented as the number of colonies per 10⁴ cells, are the mean and standard errors from three independent assays. *Statistically different (p < 0.05) from TPA-treated positive controls.

Figure 8. Inhibition of TPA-induced activation of ERKs by blackberry extract. JB6 P+ cells were pretreated with blackberry extract for 1 h and then exposed to TPA (20 ng/ml) for 30 min. Proteins (20 µg) in the cell extracts were separated by SDS-PAGE and analyzed by Western blot with phosphospecific antibody against phosphorylated sites of ERKs. Phosphorylated and nonphosphorylated proteins were detected by using the same transferred membrane blot following a stripping procedure. One representative of three similar results was shown. The histogram shows the densitometric analysis of phosphorylated protein expression normalized to total ERKs.
11). At a concentration of 40 µM, PD98059 completely blocked the colony formation of JB6 P+ cells in soft agar (Fig. 11).

Discussion

Diets rich in fruits and vegetables that contain a variety of antioxidants may reduce the incidence of cancers and other chronic diseases (1–5). In this study, we present evidence that blackberry extracts inhibited proliferation of human lung cancer cells and neoplastic transformation of JB6 P+ cells. Mechanistic studies show that blackberry extract reduces UV-induced 8-OHdG formation and MAPK-AP-1 signal ac-
tivation. These results suggest that both the inhibition of oxidative DNA damage and MAPK-AP-1 signal transduction pathway may be involved in these anticancer activities. These findings provide the evidence for a mechanism-based chemopreventive potential of blackberry extract.

It has been suggested that the use of berry products, such as strawberry and black raspberry, can attenuate initiation, promotion, and progression of carcinoma in animal models (8,10,42–45). Studies in our laboratory indicated that, besides blackberry, the extracts from strawberry and cranberry also inhibited AP-1 transactivation and cell transformation in JB6 cells (data not shown). Blackberry extract demonstrated the highest inhibitory effects among the three berry extracts evaluated. Therefore, this study was focused on the chemopreventive activity of blackberry extract.

Cell hyperproliferation is involved in cancer progression. It has been reported that oxidative stress induces the expression of several oncogenes, such as c-fos and c-jun, which enhance cell proliferation (19,41). Compared with non-neoplastic cells, cancer cells constitutively generate large basal levels of ROS that apparently function as signaling molecules in the MAPK pathway to constantly activate redox-sensitive transcription factors and responsive genes that are involved in the survival of cancer cells as well as their normal growth and replication (46,47). On the other hand, there is evidence that the antioxidant system of cancer cells is suppressed, and reducing oxidative stress may suppress the proliferation of tumor cells (48,49). From these perspectives, Toyokuni proposed the concept of “persistent oxidative stress in cancer cells” (47). Our data show that blackberry extract exposure resulted in the specific inhibition of cancer cell growth, which may be due to its antioxidant properties by perturbing the favorable redox condition in A549 cancer cells. This indicated that blackberry extract possesses the potential to inhibit cancer progression.

UVB has been proposed to be a complete carcinogen due to its direct and indirect action on cellular damage (34,50). The ROS production resulting from UVB irradiation and the subsequent oxidative DNA damage have been identified and believed to be involved in carcinogenesis (50). Beehler et al. established an in vitro model to evaluate the effect of UVB exposure on 8-OHdG production in mammalian cells (34). Our data indicated that pretreatment of cells with blackberry extract inhibited UVB-induced formation of 8-OHdG (Figs. 4A and 4B). ESR studies suggest that blackberry extract is a potent scavenger of free radicals, including OH and O2− radicals. The inhibition of 8-OHdG formation may be through the scavenging of UVB-induced ROS production as well as possible interaction between ROS and target DNA molecules. These results imply that the antioxidant effects of blackberry might play an important role in preventing or retarding environmentally induced oxidative damage and oxidant-induced diseases.

It has been suggested that AP-1 activity plays a pivotal role in cell proliferation and neoplastic transformation and is also linked to external stress (18,19,23,31,41). MAPKs, AP-1 and NF-κB are key regulators involved in immediate cellular response to oxidative stress (51). The cysteine residues present within the regulatory and binding site domains make AP-1 a very sensitive target for ROS (17,52,53). Previous studies (17,22) have indicated that ROS plays a key role in induction of AP-1 activity.

Inhibition of AP-1 activation by a variety of agents has been shown to reduce neoplastic transformation (31,32). In vivo studies in transgenic mice indicate that AP-1 transactivation is required for tumor promotion (25). The inhibition of TPA-induced cell transformation by blackberry extract might be through the attenuation of AP-1 activity. Therefore, the inhibitory effect of blackberry extract on AP-1 activation noted in this study may have a beneficial role in preventing carcinogenesis in vivo.

Studies indicate that ERKs, JNKs, and p38 kinase are key molecules activated in response to oxidant injury. Both UVB and TPA can induce ROS generation in cells (20,54). AP-1 is a downstream target of these three MAP kinases. We found that blackberry extract could scavenge OH or O2− radicals and inhibit UVB-induced phosphorylation of ERKs and JNKs, but not p38, and TPA-induced ERKs. These observations suggest that blocking of UVB- and TPA-induced AP-1-MAPK activation with fresh blackberry extract may partly be due to its antioxidant properties. In addition, it is thought that the TPA-induced AP-1 activity is mediated through the activation of PKC/MAPK/AP-1 pathway, although the detailed molecular mechanism has not been fully characterized (55). It has been reported that the polyphenol chemopreventive agent, resveratrol, may exert its effect by inhibiting PKC α activity (56). Therefore, the possible mechanisms involved in the inhibition of signaling of PKC or other pathways by the active fractions of blackberry extract warrant further studies.

In this study, we further investigated the mechanisms by which phytochemicals may prevent carcinogenesis. Black-
berry extract inhibited DNA oxidative damage, cancer cell proliferation, as well as AP-1 activation possibly by interfering with signal transduction events involving MAPKs, including ERKs and JNK, but not p38. Cell transformation studies show that blackberry extract also inhibited TPA-induced cell transformation. Our data demonstrate that blackberry extract might inhibit carcinogenesis by acting at the initiation, promotion, and progression levels. These studies open a promising area of investigation in understanding the molecular mechanisms responsible for the beneficial effects of phytochemicals in health.

Acknowledgments and Notes

We thank Dr. Lyndell Millecchia for helpful assistance with the immunocytochemical analysis. This manuscript was prereviewed in NIOSH. Address correspondence to M. Ding, Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505. Phone: (304) 285–6229. FAX: (304) 285–5938. E-mail: mid5@cdc.gov.

Submitted 5 March 2004; accepted in final form 23 June 2004.

References


