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

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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-

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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/κB; 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% CO_2 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). PD98059 was from Calbiochem (San Diego, CA). Chelex 100, $FeSO_4$, H_2O_2, xanthine, and xanthine oxidase were purchased from Sigma (St. Louis, MO). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI).

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 were 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 $O_2^{\cdot-}$ 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/m^2; 34). 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 (1×10^4) suspended in 400 µl of medium with or without blackberry extract were seeded on electrodes. The electrodes were precoated 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/κB cells was trypsinized, and 5×10^4 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% CO_2. 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/m^2) 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 (1×10^4) 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% CO_2 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 $O_2^{\cdot-}$ radicals. The Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {\cdot}OH + OH^-$) 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).

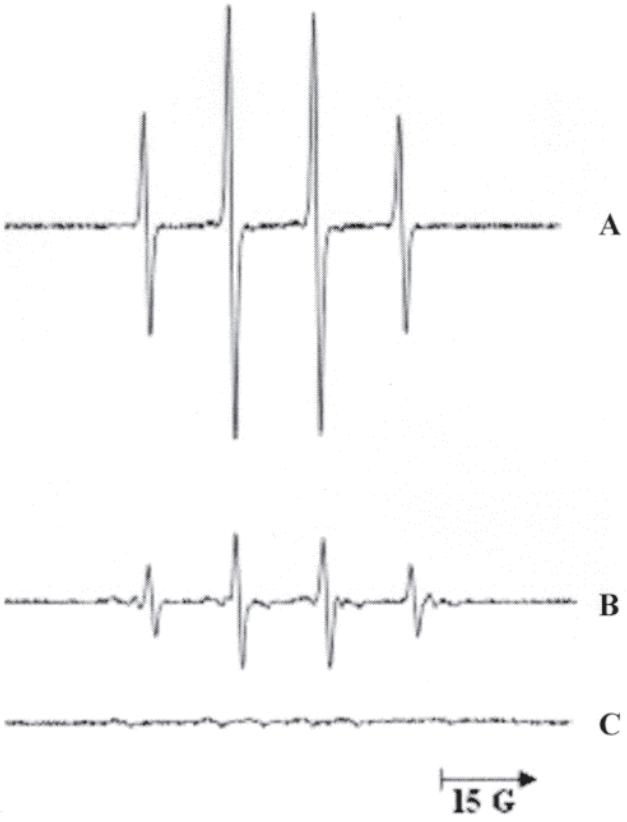

Figure 1. The scavenging effect of blackberry extract on ·OH radicals. ESR spectra was recorded after reaction initiation from a phosphate-buffered solution (pH 7.4) containing 10 mM DMPO and the following reactants: A: 1.0 mM $FeSO_4$ and 1.0 mM H_2O_2; B: 1.0 mM $FeSO_4$, 1.0 mM H_2O_2 and 0.3 mg protein/ml blackberry extract; C: 1.0 mM $FeSO_4$, 1.0 mM H_2O_2, and 3 mg protein/ml blackberry extract. The ESR spectrometer settings were: receiver gain, 2.52×10^4; time constant, 20 ms; modulation amplitude, 0.5 G; scan time, 60 s; and magnetic field, 3,480 ± 100 G.

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 $a_H^{\lambda} = 1.2$ G. These splittings are typical of the DMPO/$O_2^{\cdot-}$ spectra. The addition of blackberry extract normalized as protein concentration reduced the DMPO/$O_2^{\cdot-}$ adduct signal, demonstrating the ability of blackberry extract to scavenge the $O_2^{\cdot-}$ 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

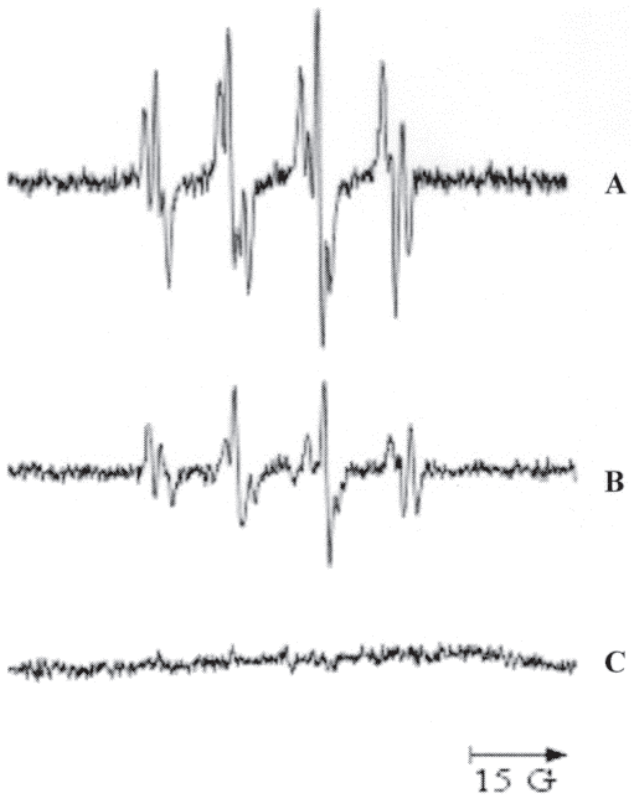

Figure 2. The scavenging effect of blackberry extract on $O_2^{\cdot-}$ radicals. ESR spectra recorded 1 min after reaction initiation from a phosphate-buffered solution (pH 7.4) containing 100 mM DMPO and the following reactants: A: 3.5 mM xanthine and 2 U/ml xanthine oxidase; B: 3.5 mM xanthine, 2 U/ml xanthine oxidase, and 0.3 mg protein/ml blackberry extract; C: 3.5 mM xanthine, 2 U/ml xanthine oxidase, and 3 mg protein/ml blackberry extract. The ESR spectrometer settings were: receiver gain, 2.52×10^4; time constant, 20 ms; modulation amplitude, 1.0 G; scan time, 60 s; and magnetic field, 3,480 ± 100 G.

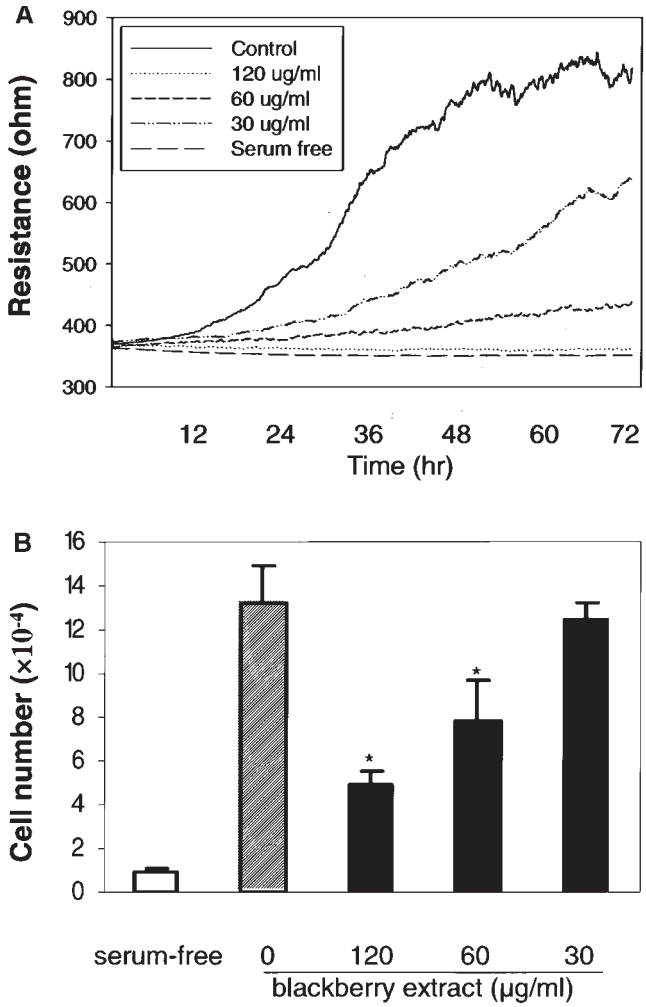

Figure 3. Inhibition of proliferation of cancer cells by blackberry extract. A: A549 cells (1×10^4) suspended in 400 µl of 10% fetal bovine serum DMEM medium with or without the indicated doses of blackberry extract were seeded on electrodes and cultured for 72 h. The ECIS resistance, indicating the cell number in the wells, was monitored for the duration of the experiment. A549 cells in serum-free medium were used as the negative control. Data are representative of two independent experiments. B: The cell number of A549 was determined using a Coulter ® Multisizer II Cell Counter at the end of experiment. Data are expressed as mean ± SE of three samples relative to the control well. *Indicates a significant inhibition of proliferation of cancer cells by blackberry extract ($P < 0.05$).

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-

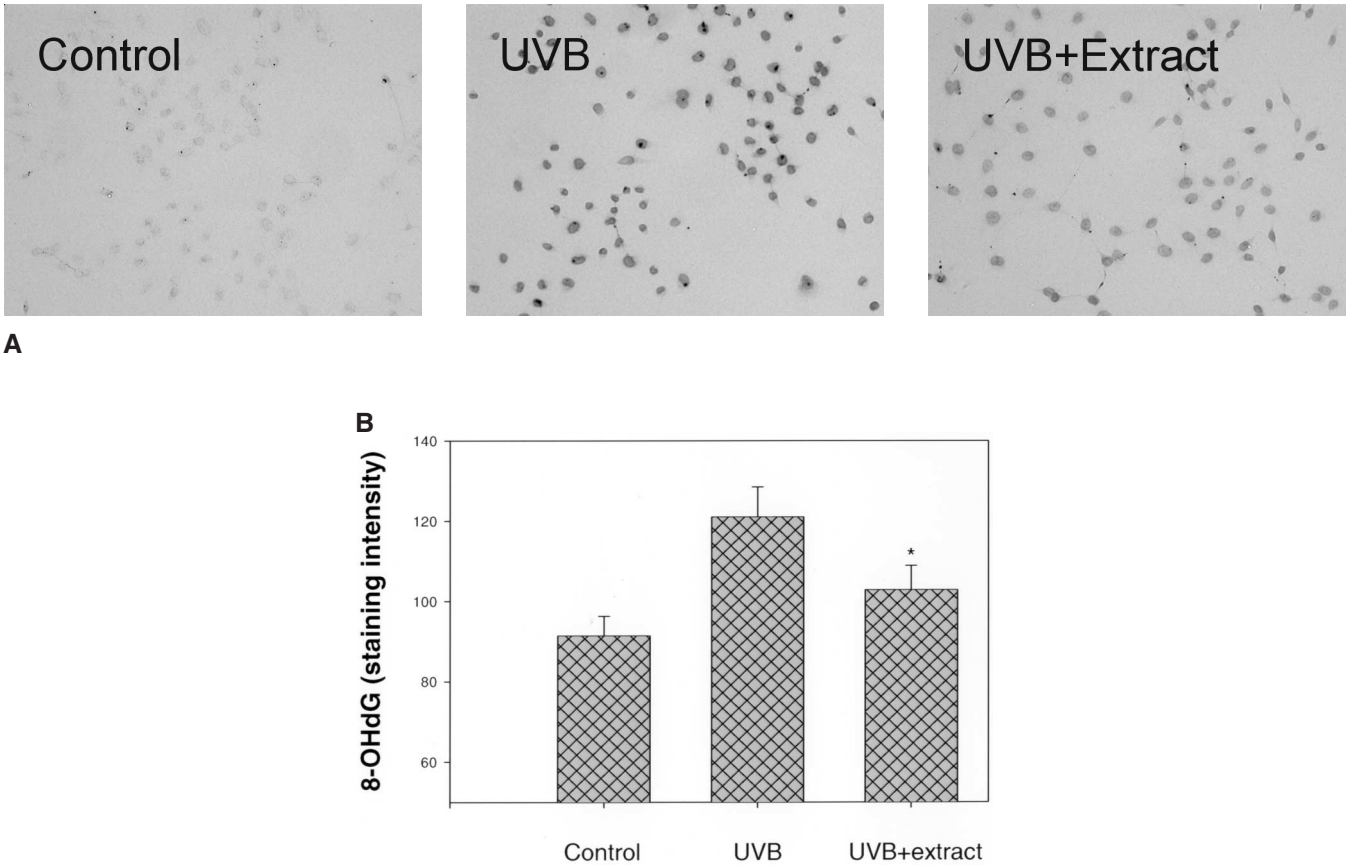

Figure 4. Inhibition of UVB-induced 8-OHdG formation by blackberry extract. A: A549 cells were seeded on four-chamber slides at a concentration of 50,000 cells per chamber and cultured for 48 h. The cells were pretreated with or without blackberry extract (0.6 mg protein/ml) for 1.5 h and then exposed to UV irradiation (6.3 kJ/m^2). The 8-OHdG formation was detected by immunohistochemical staining using a N45.1 antibody. B: Staining intensity was analyzed by computer-assisted grey-level scanning. Data are mean ± SE of six fields from two different experiments. *Indicates a significant inhibition of UVB-induced 8-OHdG formation by blackberry extract ($P < 0.05$).

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, anchorage-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.

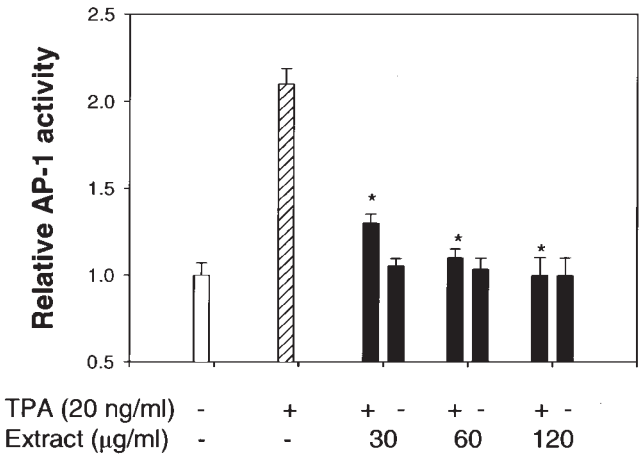

Figure 5. Blackberry extract suppresses TPA-induced AP-1 activity. JB6/AP/κB cells that stably transfected with AP-1 luciferase reporter plasmid were cultured as described in **Materials and Methods**. The cells were pretreated with or without blackberry extract at the concentrations indicated for 1 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 ($P < 0.05$).

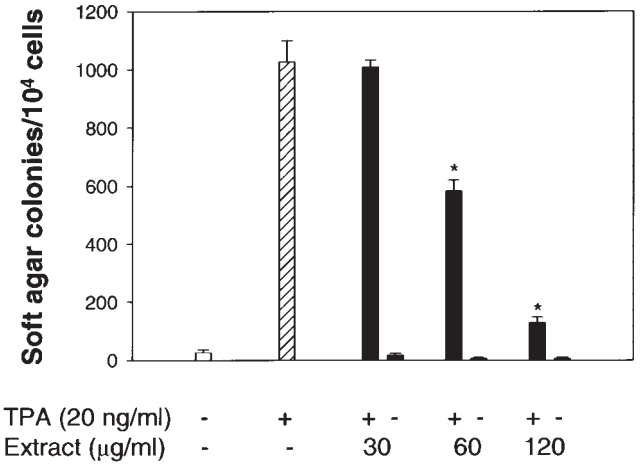

Figure 7. Inhibition of TPA-induced cell transformation by blackberry extract. JB6 P^+ cells (1×10^4) were exposed to TPA (20 ng/ml) in the presence or absence of blackberry extract in 0.33% agar for 14 days and scored for colonies at the end of the experiments. Results, presented as the number of colonies per 10^4 cells, are the mean and standard errors from three independent assays. *Statistically different ($p < 0.05$) from TPA-treated positive controls.

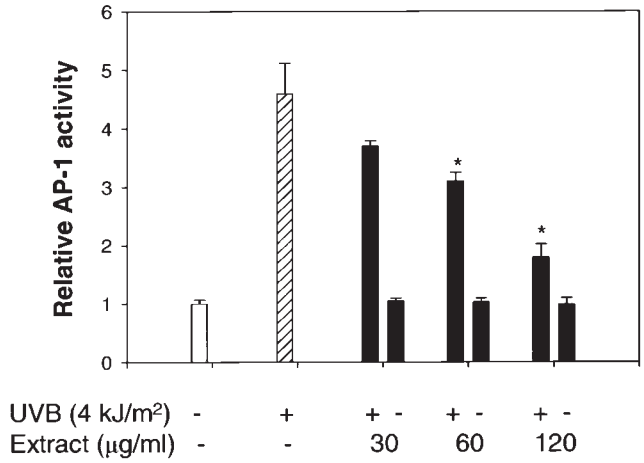

Figure 6. Blackberry extract suppresses UVB-induced AP-1 activity. JB6/AP/κB cells were cultured as described in **Materials and Methods.** The cells were pretreated with or without blackberry extract at the concentrations indicated for 1 h and then were exposed to UVB irradiation (4 kJ/m^2) and cultured 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 ($P < 0.05$).

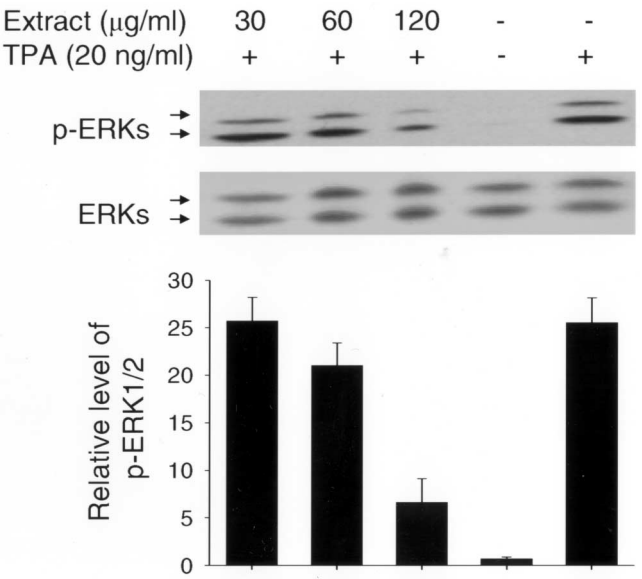

Figure 8. Inhibition of TPA-induced activation of ERKs by blackberry extract. JB6 P^+ cells were pretreated with blackberry extract for 1 h and then exposed to TPA (20 ng/ml) for 30 min. Proteins (20 μg) in the cell extracts were separated by SDS-PAGE and analyzed by Western blot with phosphospecific 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.

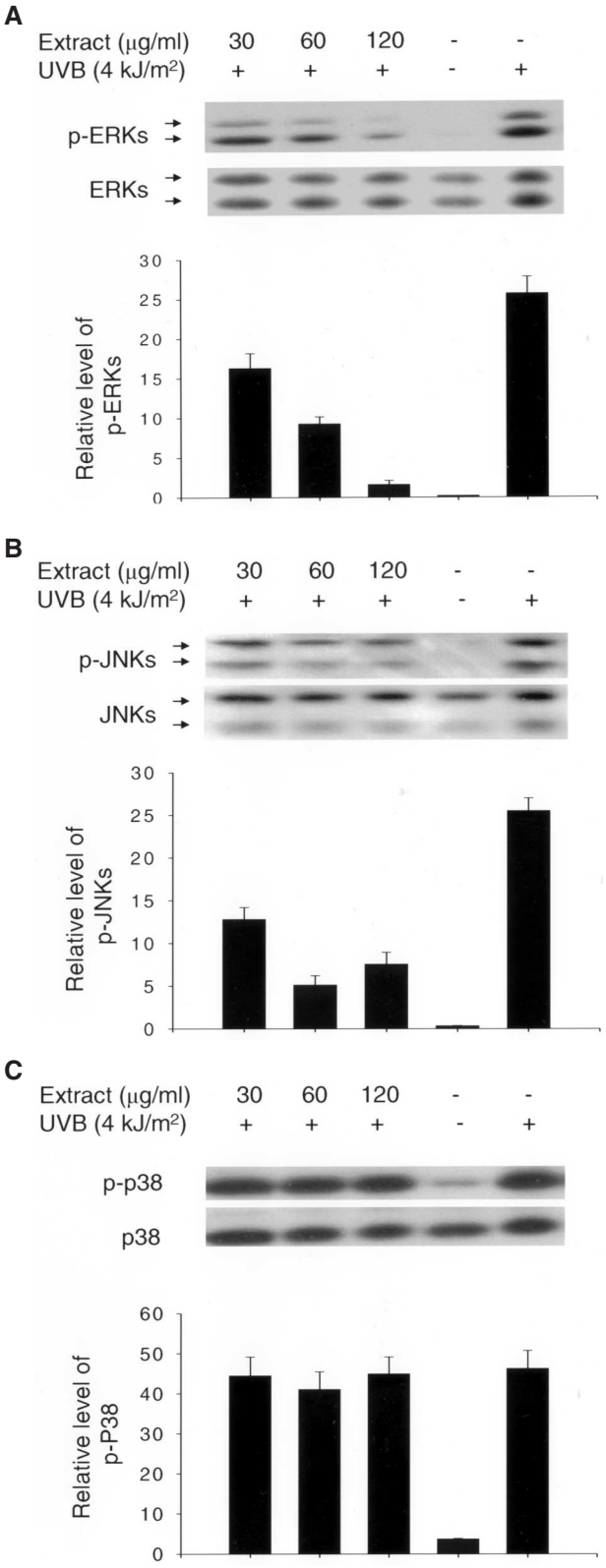

Figure 9. Inhibition of UVB-induced activation of MAPKs by blackberry extract. JB6 P+ cells were pretreated with blackberry extract for 1 h and then exposed to UVB radiation (4 kJ/m^2). Proteins (20 μg) in the cell extracts were separated by SDS-PAGE and analyzed by Western blot with phosphospecific antibody against phosphorylated sites of A: ERKs, B: JNKs, and C: p38. Data are representative of three experiments. Histograms show the densitometric analysis of phosphorylated protein expression normalized to total MAPK.

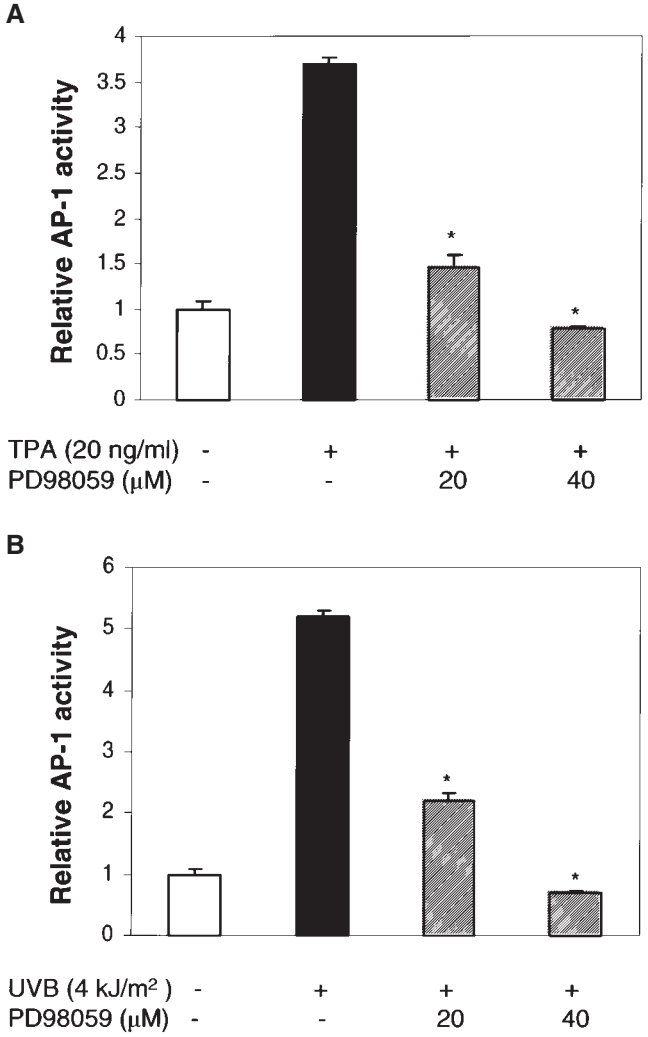

Figure 10. Inhibitory effect of PD98059 on TPA- or UVB-induced AP-1 activity. JB6/AP/κB cells were cultured as described in **Materials and Methods**. After 1 h of treatment of the cells with PD98059 at the concentrations indicated, the cells were exposed to A: TPA (20 ng/ml) and cultured for an additional 48 h or B: UVB radiation and cultured for an additional 16 h. AP-1 activity was measured by the luciferase assay. Results, presented as relative AP-1 induction normalized to the untreated control cells, are expressed as means and standard errors of three assay wells. The experiment was repeated three times. *Indicates significant different ($P < 0.05$) from TPA- or UVB-treated positive controls.

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-

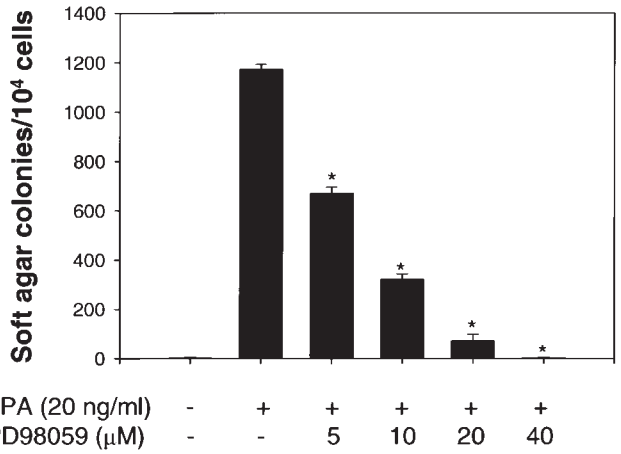

Figure 11. Inhibition of TPA-induced transformation by PD98059. JB6 P^+ cells (1×10^4) were exposed to TPA (20 ng/ml) in the presence or absence of PD98059 in 0.33% agar for 14 days and scored for colonies at the end of the experiments. Results, presented as the colony number per 10^4 cells, are the mean and standard errors from three independent assays. *Indicates significant different ($P < 0.05$) from TPA-treated positive controls.

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 are 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 $\cdot OH$ and $O_2^{\cdot -}$ 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 $\cdot OH$ or $O_2^{\cdot -}$ 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

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References

1. Vecchia CL and Tavani A: Fruit and vegetables, and human cancer. *Eur J Cancer Prev* **7,** 3–8, 1998.
2. Mukhtar H and Ahmad N: Cancer chemoprevention: future holds in multiple agents. *Toxicol Appl Pharmacol* **158,** 207–210, 1999.
3. Kobayashi M, Tsubono Y, Sasazuki S, Sasaki S, and Tsugane S: Vegetables, fruit and risk of gastric cancer in Japan: a 10-year follow-up of the JPHC Study Cohort I. *Int J Cancer* **102,** 39–44, 2002.
4. Block G, Patterson B, and Subar A: Fruits, vegetables, and cancer prevention: A review of the epidemiological evidence. *Nutr Cancer* **18,** 1–29, 1992.
5. Hong WK and Sporn MB: Recent advances in chemoprevention of cancer. *Science* **278,** 1073–1077, 1997.
6. The Ohio State University: Ohio Berry Symposium. Columbus, Ohio. Nov. 12–13, 2002.
7. Harris GK, Gupta A, Nines RG, Kresty LA, Habib SG, et al.: Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr Cancer* **40,** 125–133, 2001.
8. Stoner GD, Kresty LA, Carlton PS, Siglin JC, and Morse MA: Isothiocyanates and freeze-dried strawberries as inhibitors of esophageal cancer. *Toxicol Sci* **52,** 95–100, 1999.
9. Roy S, Khanna S, Alessio HM, Vider J, Bagchi D, et al.: Anti-angiogenic property of edible berries. *Free Radic Res* **36,** 1023–1031, 2002.
10. Xue H, Aziz RM, Sun N, Cassady JM, Kamendulis LM, et al.: Inhibition of cellular transformation by berry extracts. *Carcinogenesis* **22,** 351–356, 2001.
11. Wang SY and Jiao H: Changes in oxygen-scavenging systems and membrane lipid peroxidation during maturation and ripening in blackberry. *J Agric Food Chem* **49,** 1612–1619, 2001.
12. Marquina MA, Corao GM, Araujo L, Buitrago D, and Sosa M: Hyaluronidase inhibitory activity from the polyphenols in the fruit of blackberry (Rubus fruticosus B.). *Fitoterapia* **73,** 727–729, 2002.
13. Helser MA, Hotchkiss JH, and Roe DA: Influence of fruit and vegetable juices on the endogenous formation of N-nitrosoproline and N-nitrosothiazolidine-4-carboxylic acid in humans on controlled diets. *Carcinogenesis* **13,** 2277–2280, 1992.
14. Wang SY and Jiao H: Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *J Agric Food Chem* **48,** 5677–5684, 2000.
15. Yang CS, Maliakal P, and Meng, X: Inhibition of carcinogenesis by tea. *Annu Rev Pharmacol Toxicol* **42,** 25–54, 2002.
16. Reddy SPM and Mossman BT: Role and regulation of activator protein-1 in toxicant-induced responses of the lung. *Am J Physiol Lung Cell Mol Physiol* **283,** L1161 - L1178, 2002.
17. Ding M, Shi X, Lu Y, Huang C, Leonard S, et al.: Induction of activator protein-1 through reactive oxygen species by crystalline silica in JB6 cells. *J Biol Chem* **276,** 9108–9114, 2001.
18. Bernstein LR and Colburn NH: AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* **244,** 566–569, 1989.
19. Angel P and Karin M: The role of Jun, Fos, and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* **1072,** 129–157, 1991.
20. Kong AN, Yu R, Hebbar V, Chen C, Owuor E, et al.: Signal transduction events elicited by cancer prevention compounds. *Mutat Res* **480**–481, 231–241, 2001.
21. Primiano T, Sutter TR, and Kensler TW: Redox regulation of genes that protect against carcinogens. *Comp Biochem Physiol B Biochem Mol Biol* **118,** 487–497, 1997.
22. Ding M, Li JJ, Leonard SS, Ye JP, Shi X, et al.: Vanadate-induced activation of activator protein-1: role of reactive oxygen species. *Carcinogenesis* **20,** 663–668, 1999.
23. Dong Z, Birrer MJ, Watts RG, Matrisian LM, and Colburn NH: Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc Natl Acad Sci USA* **99,** 609–613, 1994.
24. Dong Z, Lavrovsky V, and Colburn NH: Transformation reversion induced in JB6 RT101 cells by AP-1 inhibitors. *Carcinogenesis* **16,** 749–756, 1995.
25. Young MR, Li JJ, Rincon M, Flavell RA, et al.: Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proc Natl Acad Sci USA* **96,** 9827–9832, 1999.
26. Eberhardt MV, Lee CY, and Liu RH: Antioxidant activity of fresh apples. *Nature* **405,** 903–904, 2000.
27. Lu YP, Lou YR, Xie JG, Yen P, Huang MT, et al.: Effect of dietary curcumin and dibenzoylmethane on formation of 7,12-dimethylbenz[a]anthracene -induced mammary tumors and lymphomas/leukemias in Sencar mice. *Carcinogenesis* **19,** 1697–1700, 1998.
28. Agarwal C, Singh RP, and Agarwal R: Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release. *Carcinogenesis* **23,** 1869–1876, 2002.
29. Cao G, Booth SL, Sadowski JA, and Prior RL: Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr* **68,** 1081–1087, 1998.
30. Mayne ST: Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. *J Nutr* **133,** 933S-940S, 2003.
31. Li JJ, Westergaard C, Ghosh P, and Colburn NH: Inhibitors of both nuclear factor-kappaB and activator protein-1 activation block the neoplastic transformation response. *Cancer Res* **57,** 3569–3576, 1997.
32. Ding M, Lu Y, Bowman LL, Huang C, Leonard S, et al.: Inhibition of AP-1 and neoplastic transformation by fresh apple peel extract. *J Biol Chem* **279,** 10670–10676, 2004.
33. Leonard SS, Xia C, Jiang BH, Stinefelt B, Klandorf H, et al.: Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem Biophys Res Commun* **309,** 1017–1026, 2003.
34. Beehler BC, Przybyszewski J, Box HB, and Kulesz-Martin MF: Formation of 8-hydroxydeoxyguanosine within DNA of mouse keratinocytes exposed in culture to UVB and H_2O_2. *Carcinogenesis* **13,** 2003–2007, 1992.

35. Knaapen AM, Seiler F, Schilderman PA, Nehls P, Bruch J, et al.: Neutrophils cause oxidative DNA damage in alveolar epithelial cells. *Free Radic Biol Med* **27,** 234–240, 1999.
36. Lo CM, Keese CR, and Giaever I: Impedance analysis of MDCK cells measured by electric cell-substrate impedance sensing. *Biophys J* **69,** 2800–2807, 1995.
37. Negash S, Wang HS, Gao C, Ledee D, and Zelenka P: Cdk5 regulates cell-matrix and cell-cell adhesion in lens epithelial cells. *J Cell Sci* **115,** 2109–2117, 2002.
38. Keese CR, Bhawe K, Wegener J, and Giaever I: Real-time impedance assay to follow the invasive activities of metastatic cells in culture. *Biotechniques* **33,** 842–844, 846, 848–850 passim, 2002.
39. Ding M, Dong Z, Chen F, Pack D, Ma WY, et al.: Asbestos induces activator protein-1 transactivation in transgenic mice. *Cancer Res* **59,** 1884–1889, 1999.
40. Colburn NH, Wendel EJ, and Abruzzo G: Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: mitogen-resistant variants are sensitive to promotion. *Proc Natl Acad Sci USA* **78,** 6912–6916, 1981.
41. Karin M, Liu Z, and Zandi E: AP-1 function and regulation. *Curr Opin Cell Biol* **9,** 240–246, 1997.
42. Carlton PS, Kresty LA, Siglin JC, Morse MA, Lu J, et al.: Inhibition of N-nitrosomethylbenzylamine-induced tumorigenesis in the rat esophagus by dietary freeze-dried strawberries. *Carcinogenesis* **22,** 441–446, 2001.
43. Kresty, LA, Morse, MA, Morgan, C, Carlton, PS, Lu, J, et al.: Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. *Cancer Res* **61,** 6112–6119, 2001.
44. Casto, BC, Kresty, LA, Kraly, CL, Pearl, DK, Knobloch, TJ, et al.: Chemoprevention of oral cancer by black raspberries. *Anticancer Res* **22,** 4005–4015, 2002.
45. Huang, C, Huang, Y, Li, J, Hu, W, Aziz, R, et al.: Inhibition of benzo(a)pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor kappaB by black raspberry extracts. *Cancer Res* **62,** 6857–6863, 2002.
46 Loo G: Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation. *J Nutr Biochem* **14,** 64–73, 2003.
47. Toyokuni S, Okamoto K, Yodoi J, and Hiai H: Persistent oxidative stress in cancer. *FEBS Lett* **358,** 1–3, 1995.
48. Oberley LW and Oberley TD: Role of antioxidant enzymes in cell immortalization and transformation. *Mol Cell Biochem* **84,** 147–153, 1988.
49. Lee YS, Jin DQ, Park SH, Han SY, Kim HS, et al.: 2,3,7,8-tetrachlorobenzo-p-dioxin inhibits proliferation of SK-N-SH human neuronal cells through decreased production of reactive oxygen species. *Free Radic Res* **36,** 1283–1289, 2002.
50. Peak MJ and Peak JG: Solar-ultraviolet-induced damage to DNA. *Photodermatol* **6,** 1–15, 1989.
51. Turpaev KT: Reactive oxygen species and regulation of gene expression. *Biochemistry* **67,** 281–292, 2002.
52. Sen CK and Packer L: Antioxidant and redox regulation of gene transcription. *FASEB J* **10,** 709–720, 1996.
53. Dhar A, Young MR, and Colburn NH: The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. *Mol Cell Biochem* **234–235,** 185–193, 2002.
54. Zhang X, Rosenstein BS, Wang Y, Lebwohl M, and Wei H: Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. *Free Radic Biol Med* **23,** 980–985, 1997.
55. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, et al.: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257,** 7847–7851, 1982.
56. Garcia-Garcia J, Micol V, de Godos A, and Gomez-Fernandez JC: The cancer chemopreventive agent resveratrol is incorporated into model membranes and inhibits protein kinase C alpha activity. *Arch Biochem Biophys* **372,** 382–388, 1999.