Inhibition of AP-1 and Neoplastic Transformation by Fresh Apple Peel Extract*

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Consumption of fruits and vegetables has been associated with a low incidence of cancers and other chronic diseases. Previous studies suggested that fresh apples inhibit tumor cell proliferation. Here we report that oral administration of apple peel extracts decreased the number of nonmalignant and malignant skin tumors per mouse induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in 7,12-dimethylbenz(a)anthracene-initiated mouse skin. ESR analysis indicated that apple extract strongly scavenged hydroxyl (OH) and superoxide (O2·−) radicals. Mechanistic studies showed that pretreatment with apple peel extract inhibited AP-1 transactivation induced by ultraviolet B irradiation or TPA in JB6 cells and AP-1-luciferase reporter transgenic mice. This inhibitory effect appears to be mediated by the inhibition of ERKs and JNK activity. The results provide the first evidence that an extract from fresh apple peel extract may inhibit tumor promoter-induced carcinogenesis and associated cell signaling, and suggest that the chemopreventive effects of fresh apple may be through its antioxidant properties by blocking reactive oxygen species-mediated AP-1-MAPK activation.

Chemoprevention has been acknowledged as an important and practical strategy for the management of cancer. Many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents (1–4). Animal investigations supported by epidemiological studies indicate that consumption of phytochemicals (compounds derived from plants, such as fruits and vegetables) might reduce the incidence of cancers and other chronic diseases (5–9). Constituents and micronutrients in vegetables and fruits include phytochemicals, vitamins, and minerals, which have been found to exhibit both complementary and overlapping mechanisms of chemopreventive activity in multistage carcinogenesis (10).

A recent study demonstrated that topical application of plant-derived chemicals, such as caffeine or (−)-epigallocatechin gallate, inhibit carcinogenesis and selectively increase apoptosis in UVB1-treated mouse skin (11). A previous study (12, 13) also indicated that whole fresh apple may have antioxidant and cancer chemopreventive activity. Whole-apple extracts were shown to inhibit tumor cell proliferation, and the inhibitory activity could not be attributed solely to the ascorbate content of the apples (12). It has been proposed that the consumption of whole fruits may provide the antioxidant balance needed to quench reactive oxygen species, which have been implicated in tumorigenesis (14).

Previous studies indicated that activator protein-1 (AP-1) plays a critical role in tumor promotion (15, 16). Increased AP-1 activity is associated with malignant transformation and the action of cancer-promoting agents, such as UV radiation, growth factors, and transforming oncogenes (17, 18). Blocking TPA-induced AP-1 activation has been shown to inhibit neoplastic transformation (19). Furthermore, a recent study (20), using transgenic mice, has demonstrated that AP-1 transactivation is required for tumor promotion. To elucidate the mechanism of the antitumorigenic effects of phytochemicals, we studied the effect of apple extract on UVB or TPA-induced AP-1-MAPK activation and neoplastic transformation.

Reports focusing on mechanisms for the possible chemopreventive effects of fresh fruits or vegetables are limited. In light of the important roles of ROS and AP-1 activation in tumor promoter-induced transformation and tumor promotion, we hypothesized that the anticarcinogenic effects of fresh apple may involve the inhibition of AP-1-MAPK activities due to the antioxidant properties of apples. In the present study, we evaluated the potential inhibitory effects of fresh apple extract on DMBA-TPA-induced skin papillomas in the mouse. We also evaluated the effects of these treatments on AP-1-MAPK activation induced by TPA or UV radiation and on neoplastic transformation induced by TPA in JB6 cells. The results of this investigation improve our understanding on the mechanisms by which dietary modulators and nutritional factors may control the production of ROS and result in activation of molecular signals involved in the initiation, promotion, and progression of environmentally induced neoplasia. Positive results would raise the possibility of isolating effective antioxidant compounds from natural foods for application in tumor prevention and treatment.

MATERIALS AND METHODS

Reagents—Fresh Gala apples were obtained from a local grocery store. Eagle’s minimal essential medium was purchased from Whittaker Biosciences (Walkersville, MD). Fetal bovine serum, gentamicin, and t-glutamine were from Invitrogen assay kit was obtained from Promega (Madison, WI). PhosphoPlus MAPK Antibody kits were purchased from New England Biolabs (Beverly, MA). 2’,7’-dichlorofluorescein diacetate and dihydroethidium were obtained from Molecular

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‡ The abbreviations used are: UVB, ultraviolet B; TPA, 12-O-tetradecanoylphorbol-13-acetate; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMBA, dimethylbenz[a]anthracene; AP-1, activator protein-1.
Probes (Eugene, OR). The spin trap, DMPO, was purchased from Sigma and purified by charcoal decolorization and vacuum distillation. DMPO solution, thus purified, did not contain any ESR-detectable impurities. Chelex 100 chelating resin was purchased from Bio-Rad. The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants.

Preparation of Apple Extract—Fresh apple extract was prepared from Gala apples by adding 1 ml of distilled water per g of peeled skin or flesh and blending at high speed. The blended homogenate was strained, centrifuged, and filter-sterilized. The extracts were then aliquoted and stored at -20 °C.

Cell Culture—The mouse JB6/AP-vB cell line, which was stably transfected with an AP-1 luciferase reporter plasmid (18), was cultured in Eagle’s minimal essential medium containing 5% fetal bovine serum, 2 mM l-glutamine, and 50 µg of gentamicin/ml.

Assay of AP-1 Activity in Vitro and in Vivo—A confluent monolayer of JB6/AP-vB cells was trypsinized, and 5 × 10⁶ viable cells were seeded 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 Eagle’s minimal essential medium supplemented with 0.5% fetal bovine serum for 12–24 h to minimize basal AP-1 activity. The cells were then pretreated with apple extract for 2 h followed by exposure to TPA (20 nm) or UVB radiation (4 kJ/m²) for 24 h to monitor the effects on AP-1 induction. Luciferase activity was measured by using the luciferase assay kit obtained from Promega (Madison, WI) as described previously (21).

The in vivo effect of apple peel extract was explored by using AP-1-luciferase reporter transgenic mice. The mice were given apple peel extract to drink (ad libitum), and the dorsal skin was also treated topically with apple peel extracts six times, over a period of 6 days. On the 4th day of treatment the mice were topically exposed to TPA (5 µg in 0.2 ml acetone) or UVB irradiation (10 kJ/m²). The luciferase activities were measured using dorsal skin punch biopsy samples as described previously (22, 23). Briefly, the skin tissues were biopsied by a 1.5-mm Biopsy Punch (Acuderm Inc., Ft. Lauderdale, FL) after exposure. The tissues will be dissolved in lysis buffer (from the luciferase assay kit provided by Promega) at 4 °C for 12 h, and the luciferase activity in the supernatant was measured.

Protein Kinase Phosphorylation Assay—Immunobots for phosphorylation of ERKs, JNKs, and p38 kinase were carried out as described in the protocol from Cell Signaling Technology (Beverly, MA), using phospho-specific antibodies against phosphorylated sites of ERKs, JNKs, and p38 kinase. Non-phospho-specific antibodies provided in

![Fig. 1. Effects of apple peel extract on tumorigenesis in DMBA/TPA-treated mice.](image1)

The mice were initiated with a single dose of DMBA and promoted twice a week with TPA as described under "Materials and Methods." The experimental group was given apple extract to drink (ad libitum) 2 days before DMBA treatment, and this oral administration was continued for the duration of the experiment. The number of papillomas was recorded weekly. The results are presented as the mean number of papillomas per mouse ± S.E. of 19–24 mice. * indicates a significant difference in the number of papillomas between TPA-treated groups with or without oral administration of apple extract.

![Fig. 2. External appearance of tumors.](image2)

Skin tumors were generated by DMBA and TPA treatment. A, two mice from the positive control group showing the greatest number of tumors; B, two mice from the apple-treated group showing the greatest number of tumors. Arrows indicated tumors greater than 4 mm in diameter.

![Fig. 3. Pathology of tumors.](image3)

Sections from squamous carcinomas from dorsal skin of positive control mouse (A) and squamous papillomas from apple extract-treated mouse (B). Left panels, sections ×10 of magnification. Right panels, the same sections at a higher magnification (×20). Arrows indicate vascularization in the apple peel extract-treated mouse.
each assay kit were used to normalize the phosphorylation assay by using the same transferred membrane blot.

**ESR Measurements**—All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using K$_2$CrO$_4$ and DPPH as reference standards. An EPRDAP 2.0 program was used for data acquisitions and analysis. The scavenging ability of apple extract for OH and O$_2^-$ radicals was measured by ESR.
of acetone twice a week for 20 weeks. The negative control group was treated with acetone only. The incidence of papillomas was detected by palpation, and the number of papillomas appearing on each mouse was recorded once a week. At the end of the experiment, all the animals were sacrificed by intraperitoneal injection of pentobarbital (6.5 mg/mouse). For histopathology, the largest tumors were removed and fixed in freshly prepared 4% paraformaldehyde followed by paraffin embedding and subjected to pathologic studies. 

**Statistics**—Data are presented as means ± S.E. of n experiments as noted in the figure legends. Significant differences were determined using the Student's *t* test. Significance was set at *p* ≤ 0.05.

**RESULTS**

**Inhibition of Papillomagenesis and Malignant Transformation by Fresh Apple Peel Extract**—To study the chemopreventive activity of phytochemicals, the antitumorigenic effects of fresh apple were evaluated using experimental animals. In the mouse skin model, initiation can occur as a result of a single dose of DMBA that mutationally activates H-ras (27). Promotion can be induced by repeated treatment with the tumor promoter TPA (28). The mice bearing the luciferase reporter gene were subjected to DMBA-initiation-TPA promotion. Fourteen days following initiation, the dorsal skin of the mice was exposed to TPA to cause promotion. Oral administration of apple peel extract was started 2 days before DMBA treatment and continued for the duration of the experiment. The incidences of papillomas generation are shown in Fig. 1. DMBA initiation-TPA promotion induced papillomas is an exposure-dependent lesion. Treatment of the mice with apple extract decreased the tumors per mouse at all exposure times. These differences between the apple versus non-apple groups were significant at 16 weeks after promotion. The maximum number of papillomas after 20 weeks of TPA exposure in the apple-treated mice was 12.5 ± 3.8 per mouse compared with 3.65 papillomas per mouse in the positive control group, indicating a greater than 50% inhibition of papillomagenesis by oral ingestion of apple peel extract. Two representative mice from each group showing the greatest number of papillomas are shown in Fig. 2. Tumors in the positive control group grew rapidly and were well vascularized, whereas tumors in the apple-treated group appeared growth-arrested and desiccated. The percentage of mice developing tumors was similar in both groups (data not shown).

In addition to differences in the number of tumors, we found that tumor size was significantly greater in the positive control mice compared with the apple peel extract-treated mice. At the end of the experiment, there were six tumors greater than 4 mm in diameter (arrows in Fig. 2A) in the positive control group, whereas no large tumors were found in the apple-treated mice (Fig. 2B). Malignant lesions grew rapidly and appeared ulcerated. Representative histologies of typical squamous cell carcinoma from the positive control group and squamous papillomas from the apple-treated group are shown in Fig. 3. At the end of the experiment, six tumors (2.6% in a total 228 lesion) became malignant in the positive control group, indicating a greater than 50% inhibition of papillomagenesis by oral ingestion of apple peel extract. Treatment of the mice with apple extract in a dose-dependent manner (Fig. 4A, a–e). An increase in concentration of the extract was associated with a concomitant increase of ascorbic acid-derived radical signals as indicated by asterisks. Addition of ascorbic acid also inhibited the DMPO/OH signals (Fig. 4A, f). Compar-
In Fig. 4A, c and f, the inhibition of the DMPO/OH signals was similar, but the ascorbic acid-derived signal in f was seven times greater than that in c. Furthermore, the amount of ascorbic acid-derived signal in Fig. 4A, d and e, was smaller than that in f, even though the potency of the ‘OH scavenging effect in d and e was much stronger with apple extract than that in f for ascorbic acid. These data suggest that ascorbic acid in apple peel extract plays only a partial role in the ‘OH scavenging capacity of apple extract. Fig. 4B, a, illustrates the DMPO/O$_2^*$ signal spectra generated by xanthine and xanthine oxidase. Apple peel extract exhibited a dose-dependent inhibition of O$_2^*$ radicals (Fig. 4B, b–e).

Inhibition of AP-1 Activity by Apple Extract—Because ROS are involved in AP-1 activation, and AP-1 plays a critical role in tumorigenesis induced by tumor promoters (20, 29), we conducted both in vitro and in vivo experiments to study the effects of apple peel extract on AP-1 activation. For in vitro studies, we used a mouse epidermal cell line, JB6 P$^+$ cells, stably transfected with an AP-1 luciferase reporter plasmid (19). Pretreatment of JB6 cells with various dilutions of apple extract produced a dose-dependent decrease in AP-1 activity induced by either UVB irradiation or TPA (Fig. 5, a and b). To determine whether apple peel extract had any effects on cell proliferation or apoptosis or a combination of both, serial dilutions of apple extracts from 1:10 to 1:500 were used to study the effect of apple peel extract on cell proliferation by monitoring 

**FIG. 7. Inhibitions of UVB- or TPA-induced phosphorylation of MAPKs by apple peel extract.** JB6 cells were pretreated with apple peel extract for 1 h and then were exposed to 4 kJ/m$^2$ of UVB or 20 ng/ml of TPA. The phosphorylated and non-phosphorylated ERKs, JNKS, and p38 kinase proteins in the cell lysate were assayed using a PhosphoPlus MAPKs kit from New England Biolabs. The phosphorylated proteins and nonphosphorylated proteins were detected using the same transferred membrane blot following a stripping procedure. a shows the effect of apple peel extract on UVB-induced phosphorylation of ERKs, JNKS, and p38 kinase, and b shows the effect of apple peel extract on TPA-induced phosphorylation of ERKs.

The in vivo effect of apple peel extract was explored using...
AP-1-luciferase reporter transgenic mice (26). The mice were given apple peel extract to drink, and the dorsal skin of the mice was treated topically with apple extract and then exposed to TPA or UVB irradiation. The luciferase activities were measured on dorsal skin punch biopsy samples. The apple peel extract significantly inhibited UVB-induced AP-1 activation at 24 h (Fig. 6b) and TPA-induced AP-1 activation at 72 h post-exposure (Fig. 6b). To rule out the possibility that the effect of apple extract may be due to blocking of UV radiation, we measured absorbance of UV light by apple extract and found no significant differences in absorption of UV light even at a 1:10 dilution (data not shown).

**Effects of Apple Extract on UVB- or TPA-induced MAPK Activation**—It is well known that UV irradiation results in activation of MAP kinases belonging to ERKs, JNKs, and p38 families, whereas TPA mainly activates ERKs (30–34). MAP kinases have been implicated in the activation of AP-1 (33–40). Therefore, we investigated the effect of apple peel extract on UVB- or TPA-induced MAP kinase activity by assessing the phosphorylation of the three MAPK family members. We found that pretreatment of epidermal cells with apple peel extract blocked UVB-induced phosphorylation of the MAP kinase family members ERKs and JNK (not p38) (Fig. 7a), as well as TPA-induced phosphorylation of ERKs (Fig. 7b), in a dose-dependent manner.

**Fresh Apple Extract Inhibited TPA-induced Transformation**—Previous studies (19, 20) using cell culture and transgenic animals indicate that AP-1 activation plays an important role in tumor promoter-induced neoplastic transformation. We hypothesized that one of the important anticarcinogenesis mechanisms of apples may be by suppressing tumor promotion. Therefore, we tested the effect of apple extracts on TPA-induced cellular transformation. These studies were conducted on a JB6 cells model using soft agar assays (Fig. 8). Apple peel extract inhibited TPA-induced neoplastic transformation in a dose-dependent manner. These data suggest that blocking of cell transformation by apple peel extract might be through the inhibition of AP-1 transactivation.

**DISCUSSION**

The most effective way to prevent disease is to understand the cause of an illness and then change the conditions that permit it to occur. Recent evidence has shown the importance of nutrition in delaying the aging process and in protecting against many degenerative and chronic diseases (1–8). A wide variety of phenolic compounds present in fruits and vegetables have been reported to possess anticarcinogenic and antimutagenic activity. In this study, we present evidence that oral administration of fresh apple extract inhibits the formation of keratoacanthomas and squamous cell carcinomas in the mouse skin model. The mechanism of the inhibitory effect of oral administration of apple extract on tumorigenesis appears to be via free radical scavenging action and an inhibition of AP-1-MAPK signaling. We found that fresh apple peel extract was a potent scavenger of ‘OH and O2· radicals. It also inhibited AP-1 activation and phosphorylation of MAP kinase induced by UV irradiation or TPA stimulation in an epidermal cell line and transgenic animals. Apple peel extract also blocked tumor promoter TPA-induced neoplastic transformation in cell culture. Earlier studies reported that fresh apple can inhibit cancer cell proliferation (11). The results of the present study provide further insights into mechanisms of chemoprevention by antioxidants in fresh apples.

Reactive oxygen species are generated as by-products of cellular metabolism, primarily in the mitochondria. Because ROS are partially reduced products of oxygen, they have a high chemical reactivity with a wide range of bio-macromolecules leading to lipid peroxidation and oxidation of DNA, RNA, and proteins. Because of this reactivity, oxidative stress is thought to play an important contributory role in the pathogenesis of numerous degenerative and chronic diseases, including cancer. Our study indicates that fresh apple extract is a potent scavenger of free radicals examined by ESR, including ‘OH and O2· radicals, and that ascorbic acid contained in the apple peel extract plays only a partial role in this scavenging effect. These results suggest that the antioxidant effects of fresh apples might play an important role in preventing or retarding environmentally induced oxidative damage and oxidant-induced diseases.

AP-1 is a transcription factor that is composed of Jun and Fos protein family members. AP-1 has been implicated in tumor promotion because of its ability to alter gene expression in response to tumor promoters, such as epidermal growth factor, TPA, or UV irradiation. Inhibition of AP-1 activation by a variety of agents has been shown to reduce neoplastic transformation (19). *In vivo* studies in transgenic mice indicate that AP-1 transactivation is required for tumor promotion (20). The blockade of TPA-induced cell transformation by apple extract might be through the inhibition of AP-1 activity. Therefore, the inhibitory effect of apple 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 (29, 30, 35, 36). AP-1 is a downstream target of these three MAP kinases. We found that apple peel extract could scavenge ‘OH and O2· radicals and inhibit UVB-induced phosphorylation of ERKs and JNKs but not p38 and TPA-induced ERKs. These observations suggest that blocking UVB- and TPA-induced AP-1-MAPK activation with apple peel extract may be due to its antioxidant properties.

Administration of fruits or vegetables to animals has been shown to inhibit chemically induced carcinogenesis in many animal models (37, 38). The inhibitory effects of fresh apple peel extract on TPA- or UVB-induced AP-1-MAPK signaling suggests that these pathways may be a unifying mechanism by which apples inhibit carcinogenesis and tumor growth.

Thus, in this study, we further elucidate the mechanisms by which phytochemicals may prevent carcinogenesis. Apple peel extracts inhibited AP-1 activation both *in vivo* and *in vitro*, possibly by interfering with signal transduction events involving MAP kinases, ERKs and JNK. Cell transformation studies...
show that apple peel extract also inhibited TPA-induced cell transformation. These studies open a promising area of investigation in understanding the molecular mechanisms responsible for the beneficial effects of phytochemicals on health.

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