Inhibition of Ultraviolet B-induced Activator Protein-1 (AP-1) Activity by Aspirin in AP-1-Luciferase Transgenic Mice*

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Aspirin is under consideration as a promising chemopreventative agent for human cancers. To study the usefulness of aspirin as a chemopreventative agent for UV-induced human skin cancer, we investigated the effect of aspirin on UVB-induced activator protein-1 (AP-1) activity. In the JB6 cell culture system, aspirin or sodium salicylate (SA) inhibited UVB-induced AP-1 activity in a dose-dependent manner; this inhibitory effect occurred only in cells pretreated with aspirin or SA before UVB irradiation but not cells treated with aspirin or SA after UVB irradiation. Furthermore, these inhibitory effects on UVB-induced AP-1 activity appeared to be mediated through blocking of activation of MAP kinase family members, including extracellular signal-regulated protein kinases, c-Jun N-terminal kinases, and p38. It was not due to absorption of UVB light by aspirin. In the skin of AP-1-luciferase transgenic mice, UVB irradiation induced a rapid increase in AP-1 activity, which reached the peak at 48 h post-UVB irradiation. The topical pretreatment of mouse skin with aspirin markedly blocked the UVB-induced AP-1 transactivation in vivo. These data provide the first evidence that aspirin and SA are inhibitors of UV-induced signal transduction and thus could be used as a chemopreventative agent for skin cancer.

Acetylsalicylic acid (aspirin) is one of the leading nonsteroidal anti-inflammatory drugs and is widely used as a chemopreventative agent for cancers (1–3). It was reported that patients with regular aspirin use had a reduced incidence of lung, colon, and breast cancer and/or a decreased death rate from these cancers (4–7). Furthermore, using an animal model Craven and De Rubertis reported that aspirin inhibited 1,2-dimethylhydrazine-induced colon carcinogenesis in rats injected with aspirin 1 week before carcinogen administration, whereas it had no effect when administered 4 weeks after carcinogen treatment (8). The inhibitory effect of aspirin on cancer development appears to occur in the tumor promotion step.

Because aspirin is effective in inhibiting the cyclooxygenase (COX) activity, the mechanism of aspirin's action has been explained mainly by its ability to inhibit the synthesis of prostaglandin through targeting COX (9–12). However, low doses of aspirin inhibit the synthesis of prostaglandin in vitro and in vivo (12–14), but high doses are required for its anticancer effect in vivo (15, 16). Furthermore, experiments using COX1 or COX2 gene knockout mice showed that these gene-deficient mice still exhibited the same swelling response in the ear to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) as wild-type mice (17–19). The ear swelling experiment has been used as one of the short term experiments for testing tumor promotion activity. Therefore, the inhibition of COX does not appear to be sufficient to explain all the anti-cancer effects of aspirin. More recently, we demonstrated that aspirin and sodium salicylate (SA) inhibited the transactivation of AP-1 activity in the same dose range as seen for inhibition of tumor promoter-induced cell transformation (20). Considering previous findings from different groups that tumor promoter-induced transcription of AP-1 is required for neoplastic transformation (21–26), the anti-cancer effect of aspirin may be through its blocking of tumor promoter-induced AP-1 transactivation (20).

It is well known that UVB irradiation acts both as a tumor initiator and tumor promoter, playing a major role in the development of human skin cancer (27, 28). Because transactivation of AP-1 plays a key role in tumor promotion (20, 21, 29), in the present study we investigated the effect of aspirin on UV-induced AP-1 activity in both cell cultures and AP-1-luciferase transgenic mice.

MATERIALS AND METHODS

Cell Culture and Reagents—AP-1-luciferase reporter plasmid stably transfected mouse epidermal JB6 P+ 1 cells were cultured in monolayers at 37 °C, 5% CO2 using Eagle's minimal essential medium containing 5% fetal calf serum, 2 mM l-glutamine, and 25 μg of gentamicin/ml. Fetal bovine serum (FBS) was from Life Technologies, Inc.; Eagle's minimal essential medium (MEM) was from Calbiochem (San Diego, CA); luciferase assay substrate was from Promega; aspirin was from Sigma. The phospho-specific antibodies against phosphorylated sites of Erk, p38 kinase, and JNK assay kit were from New England Biolabs.

Animals—2X TRE-luciferase reporter transgenic mice were originally established by R. A. Flavell and his co-workers (30). A C57BL/6 male mouse carrying the 2X TRE-luciferase transgene was crossed with DBA/2 (Sasco, Omaha, NE 68101) females (29). The F1 offspring were screened by testing both the basal level and TPA-induced level of luciferase activity for the presence of the AP-1-luciferase reporter gene. Males and females were housed separately in solid bottom polycarbonate cages on ventilated animal racks (~4–5 mice/cage, individualized by incisions in the ears) in temperature-, humidity-, and light-controlled conditions. Food and water were available ad libitum, and the dorsal skin of the mice was shaved every week during the experiment period. The Animal Facility at the Hormel Institute is accredited by the
Confluent monolayers of JB6 P<sup>1-1</sup> cells were trypsinized, and 8 x 10<sup>3</sup> viable cells suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. 12–24 h later, cells were starved by culturing them in 0.1% FBS MEM for 24 h prior to exposure to UVB irradiation. The cells were exposed to UVB for AP-1 induction with or without different concentrations of aspirin. After 24 h culture, the cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative AP-1 activity (26).

Erks and p38 Kinase Phosphorylation Assay—Immunoblots for phosphorylation of Erks and p38 kinase were carried out as described by the protocol of New England Biolabs using phospho-specific antibodies against phosphorylated sites of Erks and p38 kinase, respectively.

JNK Activity Assay—JNK activity was assayed as described by the protocol of New England Biolabs. In brief, JB6 C141 cells were starved for 48 h in 0.1% FBS MEM at 37 °C in a 5% CO<sub>2</sub> incubator. The cells were first treated with different concentrations of aspirin for 30 min and then exposed to UVB (4 kJ/m<sup>2</sup>) in the chamber fitted with a Kodak Kodacel K6808® filter that eliminates all wavelengths below 290 nm followed by culturing for another 20 min. The cells were washed once with ice-cold phosphate-buffered saline and lysed in 300 μl of lysis buffer/sample (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mg/ml leupeptin). The lysates were sonicated and centrifuged, and the supernatant was incubated with 2 μg of N-terminal c-Jun (1–89) fusion protein bound to glutathione-Sepharose beads overnight at 4 °C. The kinase reactions were carried out in the presence of 100 μM ATP at 30 °C for 30 min. C-Jun phosphorylation was selectively measured by Western immunoblotting using a chemiluminescent detection system and specific c-Jun antibodies against phosphorylation of c-Jun at Ser<sup>63</sup>.

Assay of AP-1 Activity in Vivo—All the mice were characterized by testing both the basal level and UVB-induced level of luciferase activity. The AP-1-luciferase reporter bearing male and female mice (6–9 weeks old) were randomly divided into four groups, including negative control group (control), UVB control group (UVB), aspirin-treated (10 μmol/mouse) group (experimental group 1), and aspirin-treated (40 μmol/mouse) group (experimental group 2). There were 13–14 mice in each group. 2 weeks after grouping, both the basal level and UVB-induced level of luciferase activities were measured with the skin biopsy tissue obtained by using biopsy punch (1.5 mm) (Acuderm, Inc., Ft. Lauderdale, FL). 2 weeks after the last punch biopsy, five topical doses of aspirin dissolved in 300 μl of acetone were applied to the dorsal skin of the mice over 8 days. The last of the five topical doses of aspirin was applied 24 h prior to exposure to UVB irradiation. The cells were exposed to UVB for AP-1 induction with or without different concentrations of aspirin. After 24 h culture, the cells were extracted with lysis buffer, and luciferase activity was measured using a luminometer (Monolight 2010). The results are expressed as relative AP-1 activity (26).

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**FIG. 2. Requirement of pretreatment of aspirin (Asp) or SA for inhibition of UVB-induced AP-1 activity in JB6 cells.** P<sup>1-1</sup> cells suspended in 5% FBS MEM were added to each well of 96-well plates. After overnight culture at 37 °C, the cells were starved by replacing the medium with 0.1% FBS MEM for 24 h. The cells were first treated with 2 mM aspirin or SA (A) or different concentrations of aspirin or SA (B) for 30 min and then sequentially were exposed to UVB (2 kJ/m<sup>2</sup>). After 24 h culture in 37 °C, 5% CO<sub>2</sub> incubator, the AP-1 activity was measured by luciferase activity assay. The results are presented as relative AP-1 activity.
given 3 h prior to UVB irradiation. Then the mice were placed in a box and exposed to 10 kJ/m² of UVB irradiation. 48 h after UVB exposure, the mice were punch biopsied to determine the effect of aspirin on UVB induction of AP-1 transactivation in the epidermis. Negative control mice were treated with acetone alone. The luciferase activity of punch biopsied epidermis was measured as described previously (26, 29). The relative AP-1 activity was presented as relative to basal level of luciferase activity of each mouse.

**Statistical Analysis**—The significance of the difference in the AP-1 activity was determined with the Student’s t test. The results are expressed as the means ± S.E.

**RESULTS**

Inhibition of UVB-induced AP-1 Activity by Aspirin or Sodium Salicylate in JB6 Cells—To determine whether aspirin and its analogue, SA, have any inhibitory effects on UVB-induced AP-1 activation, we incubated the JB6 cells with these compounds. Both aspirin and SA show marked inhibition of UVB-induced AP-1 activity (Fig. 1A). This inhibitory effect appears to be dose-dependent (Fig. 1B). The inhibitory concentration range of aspirin and SA was from 0.25 to 4 mM (Fig. 1B). To rule out the possibility that the inhibition of AP-1 activity by aspirin and SA is due to their cytotoxic effect on JB6 cells, we observed the influence of aspirin and SA on cell proliferation. JB6 cells were seeded in 96-well plates in the presence of different concentrations of aspirin or SA. After 36 h of culture, 0.5 μCi of [3H]thymidine was added to each well. The cells were harvested and incorporated [3H]thymidine was determined using a liquid scintillation counter. The results indicated that aspirin and SA at doses ranging from 0.25 to 4 mM did not show any cytotoxic effect on JB6 cells (data not shown).

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shown). To determine when aspirin or SA must be present to inhibit UVB-induced AP-1 activity, the aspirin or SA was added to cell cultures at different time points before or after cell exposure to UVB irradiation. As shown in Fig. 2, the AP-1 activity of JB6 cells induced by UV irradiation was markedly inhibited by aspirin or SA when given 1 h prior to UVB irradiation, but there was no inhibitory effect on AP-1 activity observed if the agents were given after UVB irradiation (Fig. 2).

Because normal UVB lamps also generate some UVC light, we compared the AP-1 inductions between normal UVB light and UVB light filtered with a Kodak Kodacel K6808® filter that eliminates all wavelengths below 290 nm. The results are shown in Fig. 3. AP-1 activity could be induced by either UVB light with or without filter (Fig. 3A), but a higher dose of filtered UVB light is required for similar AP-1 induction as compared with normal UVB light (Fig. 3A). The similar inhibition of AP-1 activity induced by filtered UVB light by aspirin is also observed as compared with those induced by normal UVB light (Fig. 3B).

**Inhibitory Effects of Aspirin on UVB-induced Activation of the MAP Kinase Family**—It is well known that UV irradiation results in activation of Erks and JNKs as well as p38 kinase (31–34). Our results indicated that pretreatment of cells with aspirin led to an inhibition of UVB-induced AP-1 activation. Because AP-1 has been identified as a target of MAP kinase family, including Erks, JNKs, and p38 kinase, we analyzed the inhibitory effects of aspirin on UVB-induced JNK activities and phosphorylation of Erks and p38 kinase. We found that pretreatment of cells with aspirin blocked UVB-induced JNK activity (Fig. 4A) and phosphorylation of Erks and p38 kinase (Figs. 4, B and C). These data suggested that inhibitory effects of aspirin on UVB-induced AP-1 activity are possibly modulated by blocking the activation of the MAP kinase family. To rule out the possibility that the blocking activity of aspirin on UVB-induced signal transduction is due to its absorbance of UVB light, we determined the absorbing wavelengths of aspirin.

![Fig. 5. Absorbing wavelength of aspirin to UV light. Absorbing wavelength was scanned using Beckman DU640 spectrophotometer.](image)

**Table I**

| Aspirin | UVB* | Mean ± S.D. | Inhibition |
|---------|-----|-------------|------------|
| Group 1 | Group 2 | Group 3 | % |
| 0.0     | 227  | 228  | 229    | 228.0 ± 0.8 |
| 0.25    | 238  | 236  | 235    | 236.2 ± 1.2 |
| 0.5     | 232  | 234  | 231    | 232.1 ± 1.2 |
| 1.0     | 223  | 222  | 224    | 223.0 ± 0.8 |
| 2.0     | 200  | 202  | 201    | 201.0 ± 0.8 |
| 4.0     | 171  | 170  | 173    | 171.3 ± 1.2 |

* UVB wavelength, 290–320 nm.

**FIG. 6.** UVB irradiation induces the transactivation of AP-1 in AP-1-luciferase transgenic mice. The AP-1-luciferase transgenic mice were described under “Materials and Methods.” A, 1 week after grouping, the mice were exposed to UVB (10 kJ/m²). B, the mice were exposed to UVB with the doses indicated. The dorsal skins of the mice were punch biopsied at the time indicated using biopsy punch (1.5 mm, Acuderm, Inc., Ft. Lauderdale, FL). The luciferase activity of punch biopsied epidermis was measured as described previously (26, 30) after adding 100 μl of lysis buffer overnight at 4°C. The relative AP-1 activity was presented as relative to basal level of luciferase activity of each mouse.
and examined its ability to absorb UVB light between the wavelengths of 290–320 nm, which was used in the present study. We observed that aspirin absorbs UV light in the wavelength range of 210–280 nm (Fig. 5), and aspirin did not show significant absorbance of UVB light at concentrations between 0.5–2.0 mM, which is the dose range used for blocking AP-1 activity and MAP kinase activation (Table I). Therefore, we concluded that inhibitory effects of aspirin on UVB-induced signal transduction were not through its absorbance of UVB light.

UVB Irradiation Leads to Transactivation of AP-1 in Vivo—Because the binding of AP-1 protein to DNA does not always result in an induction of transcription, the AP-1 DNA binding activity measured by gel shift assay may not correlate with AP-1 transcriptional activity in some cases (35). To test whether UVB irradiation leads to elevation of AP-1 transcription activity in vivo, we used AP-1-luciferase reporter transgenic mice. UVB (10 kJ/m²) irradiation induced a rapid increase of AP-1 activity in mouse skin within 12 h after exposure to UVB irradiation (Fig. 6A). The maximum induction of AP-1 activity (∼160-fold) was observed at 48 h post-UV irradiation (Fig. 6A, n = 7), and the activity returned to near basal levels after 10 days (data not shown). The UV dose response study indicated that optimum UVB dose for induction of AP-1 was 10 kJ/m² (Fig. 6B, n = 7); this dose for AP-1 induction is consistent with the previous dose used for skin tumor induction (36–38). These results demonstrated for the first time that UVB irradiation leads to activation of AP-1 transcription activity in AP-1-luciferase transgenic mice.

Inhibition of Transactivation of AP-1 by Aspirin in Vivo—Our preliminary results from using mouse epidermal cell line JB6 indicated that aspirin inhibits UVB-induced AP-1 activity in vitro. To test this effect in vivo, we first determined the reproducibility of UVB-induced AP-1 activation between two times of UVB exposure in the same group of AP-1-luciferase transgenic mice. The transgenic mice were punch biopsied (1.5 mm) twice on the dorsal skin to determine the basal luciferase activity within a 3-week interval. 1 week after each of these punches, the mice were irradiated with 10 kJ/m² of UVB, and 48 h later the same mice were punch biopsied to determine the UVB-induced AP-1 activity. The UVB-induced levels of luciferase activity from the two time points in the same group of mice were similar (n = 13, p > 0.05) (Fig. 7A). These data suggested that induction of AP-1 activity by UVB in the same transgenic mice can be used as a control. Because chemoprevention of cancer is a long term process, the aspirin inhibition of AP-1 induction by UVB irradiation was determined following five applications of aspirin to mouse skin over 8 days. Multiple treatments of mouse dorsal skin with aspirin resulted in significant inhibition of UVB-induced AP-1 activity at both 10- and 40-μmol doses/mouse as compared with either UVB control group mice or experimental control mice (Fig. 7B) (n = 13, p < 0.05). These results strongly demonstrate that application of
aspirin not only blocks UVB-induced AP-1 activity in a cell culture model but also inhibits AP-1 transactivation in vivo.

**DISCUSSION**

Both epidemiological and clinical studies have indicated that aspirin and related compounds have considerable potential as chemopreventative agents for cancers. Because several lines of evidence have suggested a causal role for AP-1 during tumor promotion, here we investigated the inhibitory effect of aspirin on UVB-induced AP-1 activity in both in vitro and in vivo models. Pretreatment of cells with aspirin significantly blocked the UVB-induced AP-1 transactivation and JNK activation, as well as phosphorylation of Erks and p38 kinases. This inhibition by aspirin was only observed when the aspirin treatment of the cells preceded UVB irradiation but not post-UVB irradiation. The negative results for the absorbance of UV light by aspirin suggested that inhibitory effects of aspirin on UVB-induced signal transduction is due to its pharmacological effects and not due to UVB absorbance. Topical application of aspirin on AP-1 reporter transgenic mice markedly inhibits UVB-induced AP-1 transactivation. Thus, these results, together with previous findings that UVB-induced AP-1 transactivation is a major mediator of development of human skin cancer, indicated that aspirin may serve as a chemopreventative drug for skin cancers induced by UV irradiation.

Exposure of mammalian cells to UV irradiation not only causes DNA damage, resulting in either cell death or somatic mutation, but also induces specific cell reactions, including induction of transcriptional activation such as AP-1 and nuclear factor-kB (39–41). The signal transduction pathways leading to transcription factor activation have been extensively studied in the last several years. It is believed that stress-related signals, such as UV light, induce the activation of MAP kinase pathways (Erks, JNKs, and p38). AP-1, consisting of Jun/Fos dimers, is a downstream target of these three MAP kinase members (42). We have reported that the anti-tumor promotion effects of aspirin are mediated by blocking tumor promoter-induced AP-1 activation (20). We have found that aspirin inhibited UVB-induced AP-1 activity in a dose-dependent manner, and this inhibitory effect appears to be through blocking UVB-induced activation of Erks and JNKs as well as p38 kinase. Interestingly, it appears that the signal transduction pathway leading to activation of AP-1 by UVB irradiation is different from that induced by TPA or epidermal growth factor because although aspirin inhibits UVB signals through MAP kinase-dependent pathway, it blocks TPA- or epidermal growth factor-induced signaling through a MAP kinase-independent pathway (20). These findings agree with the Schwengel et al. report that SA inhibited tumor necrosis factor-induced Erks activation but did not affect Erk activation by epidermal growth factor under the same condition (43).

Solar irradiation has been associated with nonmelanoma skin cancer for centuries (44). Epidemiological investigation found that outdoor laborers, such as fisherman and gardeners, are more prone to skin cancer, particularly in the sun-exposed areas of the body (44). The incidence of skin cancers in Caucasians has been reported to be higher in populations living closer to the equator (44). This hypothesis was further supported by the finding that the degree of intrinsic skin pigmentation was inversely correlated with the incidence of cutaneous cancer (44). Further studies indicated that the UV spectrum in solar light is responsible for its carcinogenic effect. The UV spectrum is subdivided into three ranges, including short (UVC, 200–280 nm), long (UVA, 320–400 nm), and mid-wavelengths (UVB, 280–320 nm). UV irradiation acts as both a tumor initiator and promoter in animal models (27, 28). It was generally accepted that the initiating effect of UV light is through the absorption of energy of UV light by DNA of irradiated cells and resulting in formation of pyrimidine dimers, whereas the activation of membrane-related signal transduction pathway, leading to activation of AP-1, is responsible for UV tumor promotion action (44–51). AP-1 was first considered as a mediator of tumor promotion by its ability to alter gene expression in response to tumor promoters, such as epidermal growth factor, TPA, or UV irradiation (52). It was found that AP-1 activity is progressively elevated in mouse epidermal JB6 cell variants representing various stages of tumor promotion (53). In contrast, blocking of AP-1 activity by either pharmacological inhibitors, such as flunisolone acetonide, retinoic acid, or molecular biological inhibitors, including dominant negative c-Jun (TAM67) and dominant negative phosphatidylinositol-3 kinase (AP58), impaired neoplastic transformation (21, 25, 26, 54, 55). Moreover, acquisition of a tumor promotion-resistant phenotype is associated with a loss of responsiveness to tumor promoter-induced AP-1 activation (56), and rescuing the response to tumor promoter in AP-1 activation by introducing wild-type extracellular signal-regulated protein kinase-2 (Erk2) converts tumor promotion-resistant phenotype to tumor promotion-sensitive phenotype.2 This notion was further supported by our very recent finding that the retinoid SR11302, an AP-1 inhibition-specific retinoid, markedly inhibits the TPA-induced papilloma formation and AP-1 activation in 7,12-dimethylbenz(a)anthracene-initiated AP-1-luciferase transgenic mouse skin, whereas repeated applications of another retinoid, SR11235, a transcriptional activator of retinoic acid response element, does not inhibit papilloma formation and AP-1 activation (26).

In light of the role of AP-1 activation in chemical- and photo-carcinogenesis, we investigated the possible usefulness of aspirin in chemoprevention of UV-induced skin cancer by determining its inhibitory effect on UVB-induced AP-1 activity in both a cell culture model and in an AP-1-luciferase transgenic mouse model. The results indicated that pretreatment of cells and mouse skin with aspirin blocks UV-induced AP-1 transactivation effectively in both models. It is suggested that aspirin may be used as a chemoprevention agent for nonmelanoma skin cancers.

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