Molecular mechanisms of chemoprevention: the role of signal transduction pathways

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Signal transduction pathways in the tumor promotion process

Mitogenic stimulation is likely to be an important component of tumor promotion. However, that alone is not sufficient for transformation, and changes in gene expression are required to avoid growth regulation or differentiation. In general, alterations in the transcription of a specific set of cellular genes are mediated by specific regulatory DNA binding proteins or transcription factors that regulate gene expression directly by binding to specific DNA sequences in promoter regions (1-9). The expression of genes transcriptionally induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and other tumor promoters such as UV irradiation are thought to be required in tumor promotion (4-10).

UV irradiation from the sun is the most important environmental carcinogen and is responsible for a high incidence of non-melanoma skin cancers (NMSC) in humans. Mechanistically, UV induces both genotoxic effects, such as DNA damage, and mutations, including p53 or ras mutations. Such a DNA-damaging effect is proposed as the mechanism of UV-induced initiation and UV-induced signal transduction is believed to be related to tumor promotion. UV-enhanced gene expression mediated by the initiation of a transcriptional induction response is known as the "UV response". Two transcription factors are implicated in the "UV response", AP-1 and NFκB (6).

The 7,12-dimethylbenz(a)anthracene-TPA mouse skin model is a well-characterized model for the study of tumor promotion *in vivo*. Our understanding of multi-stage carcinogenesis is based largely on data generated from this model. Recently, we showed that AP-1 is induced (11) and that inhibition of AP-1 also represses tumor promotion in this model.

The JB6 mouse epidermal cell system of clonal genetic variants that are promotion-sensitive (P^+) or promotion-resistant (P^-) allows the study of genetic susceptibility to transformation, promotion and progression at the molecular level (5, 10-17). The P^-, P^+ and transformed (Tx) variants are a series of cell lines representing "earlier-to-later" stages of preneoplastic-to-neoplastic progression. P^- variants gain P^+ phenotype upon transfection with mutated p53 (18, 19). The P^+ cells gain Tx phenotype irreversibly upon treatment with TPA, epidermal growth factor (EGF) or other tumor promoters with c-Jun overexpression (20, 21). Transformed variants grow under anchorage-independent (AI) conditions and are tumorigenic in nude or BALB/c mice in the absence of tumor promoting conditions. One of the few molecular events known to distinguish P^- or P^+ cellular responses to tumor promoters is the activation of AP-1-driven transcriptional activity in P^+ cells but not in P^- cells (10-17).

This model is a well-developed cell culture system for studying tumor promotion and anti-tumor promotion *in vitro*. We generated stable AP-1-luciferase or NFκB-luciferase transfectants in JB6 cells and used these cell lines for the study of signal transduction pathways for tumor promotion.

Through comparison of promotion sensitive (P^+) and promotion-resistant (P^-) derivatives of the mouse epidermal JB6 cell line, we found that transcriptional factor AP-1 plays a critical role in tumor promotion (Figure 1) (5, 10, 12). AP-1 is only activated in P^+ cells, but not in P^- cells (13). Furthermore, blocking the tumor promoter-induced AP-1 activity inhibited neoplastic

transformation (5, 10). Overexpression of a dominant negative mutant of Jun caused carcinoma cells to lose their tumorigenicity in nude mice (14, 15). Inhibition of AP-1 activity in transformed JB6 RT101 cells caused reversion of tumor phenotype (16, 17). Through the mechanism of protein-protein interaction of Jun with one retinoic acid receptor, retinoids block AP-1 activity in these cells. Because only those retinoids that inhibit AP-1 activity also inhibit tumor promoter-induced transformation, and the retinoic acid response element (RARE) activation-specific retinoid did not inhibit tumor promoter-induced transformation, we believe that inhibition of tumor promoter-induced transformation is an AP-1-dependent and not a RARE-dependent event. We used a more specific AP-1 inhibitor, dominant negative mutant of c-Jun (TAM67) in JB6 cells. All stable TAM67 transfectants blocked TPA-induced AP-1 activity and transformation (10). Because AP-1 DNA binding is not always correlated with AP-1 transcription activity, the best way to study AP-1 activity *in vivo* is to use transgenic animals expressing an AP-1 reporter gene such as luciferase. We used an AP-1 luciferase transgenic mouse model to study the role of AP-1 activity in tumor promotion *in vivo*. Our data indicated that AP-1 inhibitory retinoids, but not RARE activation-specific retinoid, repressed skin tumor promotion (11).

As discussed above, tumor promoters (e.g., TPA, TNFα or UV irradiation) also induce activation of transcription factor NFκB in many cell systems. In JB6 cells, inhibition of NFκB activation by antisense or pentoxifylline also blocks tumor promoter-induced cell transformation (22).

Three classes of MAPKs are known and they include extracellular-signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 kinases (Figure 1) (23-26). The activation of MAPKs may occur by translocation to the nucleus, where these kinases phosphorylate target transcription factors such as AP-1 (25-28). ERKs are believed to be strongly activated and to play a critical role in transmitting signals initiated by TPA and growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (29-30). On the other hand, JNKs/SAPKs and p38 kinases are potently activated by various forms of stress, including UV irradiation (26, 31). However, the activation of these pathways is not mutually exclusive. For example, heat shock and UV irradiation partially activate the ERKs cascade and EGF partially activates the JNKs/SAPKs pathway (25, 30). The UVC-induced activation of the AP-1 complex involves altered phosphorylation of the c-Jun protein. In unstimulated cells, the c-Jun protein is phosphorylated in the C-terminal half on a tryptic peptide, 227-252, located just upstream of the basic region of the DNA binding domain (32). UVC irradiation of cells causes enhanced DNA binding activity of Jun and the net phosphorylation of peptide 227-252 is decreased. The mechanism for the UVC-induced decrease in phosphorylation of this basic domain near the DNA binding region is not known. All of the stimuli including UVC that increase the transactivating potential of Jun cause hyperphosphorylation of two amino acids, serine-63 and serine-73 on the N-terminus (33-35). Hyperphosphorylation of serines 63 and 73 of c-Jun is suggested to prolong the interaction between Jun and p52/54 intermediary factors leading to more stable assembling of the pre-initiation complex and enhanced initiation of transcription (33).

Because different tumor promoters stimulate the activation of distinct MAP kinases, we proposed that the tumor promotion process induced by these different tumor promoters might

depend on specific MAP kinase pathways. Indeed, we found that ERK is required for TPA- or EGF-induced cell transformation in JB6 cells (36-37). Shortage of ERK is responsible for resistance to AP-1 transactivation and transformation in JB6 P^- cells (36). Blocking MAP kinase activation by dominant negative mutant (DMN) ERK1 blocks TPA-induced AP-1 transactivation in JB6 P^+ cells and DNM-ERK also block arsenic-induced cell transformation (38). On the other hand, JNK activation is required for JB6 cell transformation induced by TNFα but not by TPA (39).

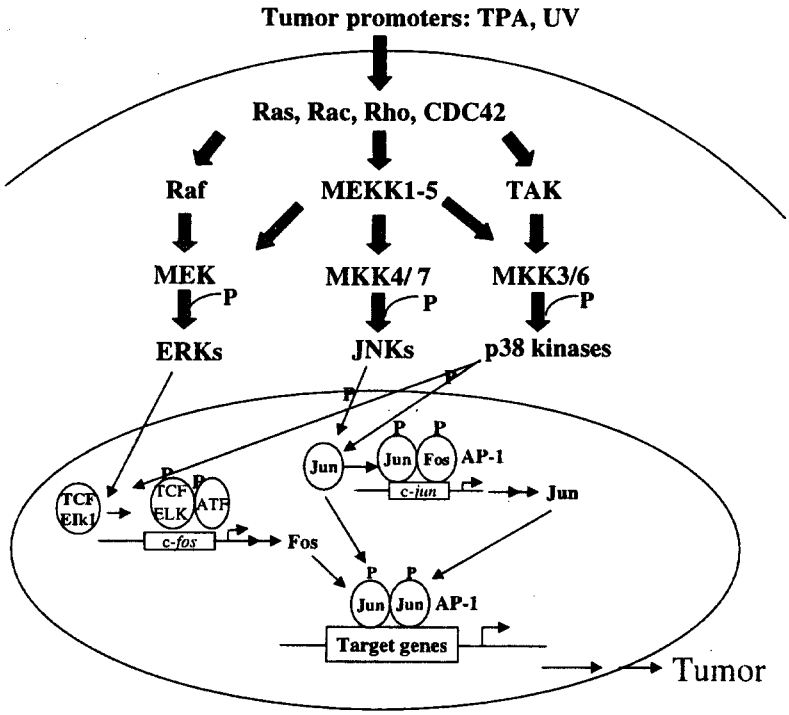

Figure 1. Tumor promoter-induced signal transduction

Effects of chemopreventive agents on tumor promoter-induced signal transduction pathways

AP-1 and NFκB signal transduction pathways are known to be important in tumor promoter-induced transformation and tumor promotion. Both AP-1 and NFκB are activated by various tumor promoters. Therefore, the inhibitory effect of chemopreventive agents on the tumor promoter-induced signal transduction leading to the activation of AP-1 or NFκB may be important in the anti-tumor promotion activity of these compounds. We, therefore, investigated the inhibition of these signal transduction pathways as molecular mechanisms for the anti-tumor promotion activity of chemopreventive agents.

Aspirin suppresses cell transformation and AP-1 activation

Aspirin, along with its analgesic-antipyretic uses, is now also being considered for prevention of cardiovascular disease, cancer and treatment of human immunodeficiency virus infection. Although many of aspirin's pharmacological actions are related to its ability to inhibit prostaglandin biosynthesis, some of its beneficial therapeutic effects are not completely understood. We have investigated the anti-carcinogenesis effect of aspirin at the molecular level. Aspirin and aspirin-like salicylates inhibited the activation of AP-1 in the same dose range as seen for the inhibition of tumor promoter-induced transformation (40). The inhibition of AP-1 and tumor promoter-induced transformation in JB6 cells occurs through a prostaglandin-independent and an Erk1- and Erk2-independent pathway (40). The elevation of H^+ concentration may involve the inhibition of AP-1 and transformation in this cell culture model. The inhibitory effects on the activation of AP-1 activity by aspirin and aspirin-like salicylates may further explain the anti-carcinogenesis mechanism of action of these drugs. To study the usefulness of aspirin as a chemopreventive agent for UV-induced human skin cancer, we investigated the effect of aspirin on UVB-induced 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 in cells treated with aspirin or SA after UVB irradiation (41). 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 kinases. It was not due to absorption of UVB light by aspirin (41). In the skin of AP-1-luciferase transgenic mice, UVB irradiation induced a rapid increase in AP-1 activity, which reached a 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* (41). These data provide the first evidence that aspirin and SA are inhibitors of UV-induced signal transduction and thus could be used as chemopreventive agents for skin cancer.

EGCG and theaflavins inhibit tumor promoter-induced cell transformation, AP-1 activity and NFκB activity

EGCG and theaflavins are the major active tea polyphenols in green tea and black tea, respectively (39). EGCG and theaflavins inhibited EGF- or TPA-induced cell transformation in a dose-dependent manner. At a dose range (5-20 μm) that inhibited cell transformation, EGCG and theaflavins also inhibited AP-1-dependent transcriptional activity (42). Our results showed that EGCG or theaflavins inhibited TPA-induced AP-1 DNA binding activity. Furthermore, these two tea compounds also inhibited TPA- or EGF-induced JNK activation. Therefore, the inhibition of AP-1 transactivation by EGCG and theaflavins occurs through an inhibition of AP-1 DNA binding activity and a JNK-dependent pathway (42).

The UVB portion of solar light is an important causative factor for human skin cancers and UV-induced signal transduction pathways play a critical role in tumor promotion. We therefore investigated the effect of EGCG and theaflavins on UVB-induced AP-1 and NFκB

activity. Pretreatment of JB6 cells with EGCG or theaflavins inhibited UVB-induced AP-1 and NFκB activity. At a similar concentration range (1-20 μM), EGCG and theaflavins also inhibited UVB-induced phosphorylation of P44/42 MAP kinase, while phosphorylation of p38 kinases was not affected. Furthermore, IκBα phosphorylation, a critical step for the activation of NFκB transactivation, was significantly inhibited by EGCG and theaflavins.

Since DNA binding activity measured by gel-shift assay does not always correspond with AP-1 or NFκB transcription activity, the best way to study transcription activity *in vivo* is by using a transgenic mouse model containing a reporter gene. Recently our laboratory used a B6D2 transgenic mouse expressing the luciferase reporter gene under the control of four TREs (AP-1 binding sequences) to study the role of AP-1 activity in tumor promotion and progression (43). In mouse skin epidermis, UVB irradiation induced a nearly 40-fold increase in luciferase activity, as compared with acetone-treated controls. Treatment with topical EGCG reduced this UVB-induction of AP-1 transactivation activity by 60%. By inhibiting AP-1 activity in UVB-irradiated mouse skin, EGCG may be preventing non-melanoma skin cancer at the level of tumor promotion (43).

Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway

Resveratrol, a plant constituent enriched in the skin of grapes, is one of the most promising agents for the prevention of cancer. However, the mechanism of the anti-carcinogenic activity of resveratrol is not well understood. Our data offer a possible explanation of its anti-cancer effect (44). Resveratrol suppressed tumor promoter-induced cell transformation and markedly induced apoptosis, transactivation of p53 activity and expression of p53 protein in the same cell line and at the same dosage. Also, resveratrol-induced apoptosis occurred only in cells expressing wild-type p53 ($p53^{+/+}$), but not in p53-deficient ($p53^{-/-}$) cells, while no difference in apoptosis induction was observed between normal lymphoblasts and sphingomyelinase-deficient cell lines. These results demonstrate for the first time that resveratrol induces apoptosis through activation of p53 activity, suggesting that its anti-tumor activity may occur through the induction of apoptosis (44).

Inositol hexaphosphate ($InsP_6$) inhibits cell transformation and AP-1 activation by targeting phosphatidylinositol-3 kinase

$InsP_6$, also known as phytic acid, is a ubiquitous compound found in the plant kingdom (45, 46) but it is also a component of mammalian cells found at concentrations between 10 and 100 μM in both resting and stimulated cells (47). Previous studies elucidated the primary functions of $InsP_6$ (48). $InsP_6$ is suggested to regulate heart rate and blood pressure, stimulate Ca^{2+} influx, bind to the clathrin assembly protein, and inhibit L- and P-selection function *in vitro* and inflammation *in vivo* (48, 49). Studies by Shamsuddin *et al.* (48) and others (50, 51) demonstrated a striking anticarcinogenic effect of $InsP_6$ and myo-inositol. $InsP_6$ was shown to be

both chemopreventive and chemotherapeutic in rodent colon and mammary carcinogenesis models, as well as in transplanted fibrosarcoma models (52).

We have investigated the influence of $InsP_6$ on tumor promoter-induced cell transformation in JB6 cells. The results indicated that $InsP_6$ markedly blocks epidermal growth factor-induced phosphatidylinositol-3 (PI-3) kinase activity in a dose-dependent manner in JB6 cells and directly *in vitro*. Blocking PI-3 kinase activity by $InsP_6$ profoundly impairs EGF- or phorbol ester-induced JB6 cell transformation and ERK activation, as well as AP-1 activation. These results provide the first evidence that the molecular mechanism of $InsP_6$ antitumor promotion effect targets and blocks PI-3 kinase activation and demonstrate that PI-3 kinase can serve as a molecular target for the development of cancer chemopreventive agents (53).

Essential role of p53 in phenethyl isothiocyanate-induced apoptosis

Phenethyl isothiocyanate (PEITC), a natural product that occurs in certain cruciferous vegetables, is among the most effective cancer chemopreventive agents known. Mechanistic studies indicate that the chemopreventive activity of PEITC is associated with its favorable modification of carcinogen metabolism and its induction of apoptosis. We found that PEITC blocks tumor promoter TPA- or EGF-induced cell transformation in mouse epidermal JB6 cells, and this inhibitory activity on cell transformation corresponds with induction of apoptosis (54). Most importantly, apoptosis induction by PEITC occurs through a p53-dependent pathway. This was demonstrated not only by results showing that PEITC induced p53 protein expression and p53-dependent transactivation, but also by results showing that PEITC induced apoptosis in $p53^{+/+}$, but not in p53-deficient ($p53^{-/-}$) cells. In contrast, PEITC induced apoptosis in cells with both normal or deficient sphingomyelinase activities. Our results demonstrate for the first time that p53 elevation is required for PEITC-induced apoptosis, which may be involved in its cancer chemopreventive activity (54).

Inhibition of tumor promotion by retinoic acid is through the inhibition of the AP-1 pathway

Retinoic acid, a derivative of vitamin A, is a potential chemopreventive agent. As was stated earlier, because AP-1 DNA binding activity is not always correlated with AP-1 transcription activity, the best way to study the AP-1 activity *in vivo* is to use transgenic animals expressing an AP-1 reporter gene such as luciferase. We used an AP-1 luciferase transgenic mouse model to study the role of AP-1 activity in tumor promotion in animals. Our data indicated that AP-1 inhibition specific retinoids, but not RARE activity-specific retinoids, showed an inhibitory effect on skin tumor promotion (11). These results add to the growing amount of evidence that targeting AP-1 is feasible for prevention of cancer.

Conclusions

Recently chemoprevention of cancer has been suggested to be facilitated by isolation and supplement of various anti-cancer food factors such as minerals, vitamins, tea polyphenols, and other food-related chemicals. We used cell culture, transgenic mice and knockout mice models to examine the chemopreventive effects of these food factors at the molecular level. We found that these food factors targeted different molecules in specific signal transduction pathways induced by tumor promoters (Figure 2). (1) Tea polyphenols, EGCG and theaflavins inhibit EGE or TPA-induced JB6 cell transformation. At the same dose range that inhibited cell transformation, EGCG and theaflavins inhibited AP-1 activation. These compounds also inhibited UVB-induced AP-1 and NFκB-dependent transcriptional activation. (2) Resveratrol inhibited cell transformation by inducing apoptosis, mediated through JNK pathways. (3) $InsP_6$ inhibited TPA- or EGF-induced transformation and signal transduction through its effects on PI-3 kinase. (4) Phenethyl isothiocyanate (PEITC) inhibited cell transformation, correlating with the induction of apoptosis. An elevation of p53 is required for PEITC-induced apoptosis. (5) Retinoids inhibited tumor promoter-induced cell transformation and tumor promotion in transgenic mice through the inhibition of AP-1 action but not through the activation of retinoic acid response element (RARE). (6) Aspirin inhibited cell transformation through an AP-1-dependent pathway. Figure 2 indicates a summary of our results showing the effects of food factors on different signal transduction pathways (Figure 2).

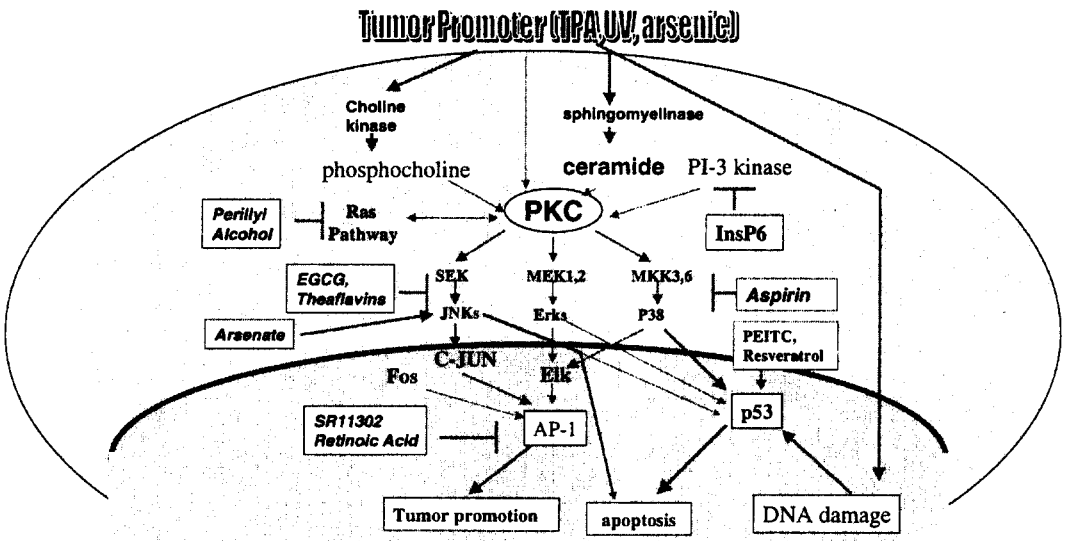

Figure 2. Chemopreventive agents target different molecules in signal transduction pathways

Prevention of cancer and other related diseases is the most challenging task in the 21st century. We need more knowledge regarding the relationship between foods and disease. Advances in genetics and molecular biology will serve as powerful tools to elucidate details of interaction between genes and food factors to determine the effect of food factors on signal transduction pathways. These advances will further define the responsible genes, proteins and pathways for the induction and suppression of carcinogenesis and aid in drawing detailed pictures of food factors and their molecular targets for prevention and treatment of cancer.

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