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Inhibition of epidermal growth factor-induced cell transformation by tannins

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Abstract

The mouse epidermal JB6 cell system is a well developed model for studying tumor promotion, and the JB6 Cl 41 promotion sensitive (P+) cell line, in which transformed colonies are induced by epidermal growth factor (EGF), was used to test the anti-tumor promoting effect of seven tannins and two triterpenoids. We found that six tannins, ellagitannins (compounds **1**, **2**, **3** and **4**) and chromone gallates (compounds **6** and **7**), significantly blocked EGF-induced cell transformation in a concentration-dependent manner. The inhibition of cell transformation by the tannins was not due to growth inhibition. The ellagitannins, but not the chromone gallates, significantly attenuated EGF-induced activator protein 1 (AP-1) activation, a transcription factor. Compounds **1** and **3**, among the ellagitannins analysed, inhibited the EGF-induced phosphorylation of extracellular-signal regulated protein kinases and p38 kinases, which regulate AP-1 activation. On the other hand, compounds **3** and **4** suppressed EGF-induced phosphatidylinositol 3-kinase (PI3K) activation. In addition, all tannins that blocked cell transformation markedly inhibited EGF-induced activation of Akt, a downstream effector of PI3K. Because signal-transduction pathways, including AP-1 and PI3K pathways, have been focused as prime targets for chemopreventive phytochemicals, our results suggest that inhibition by tannins of EGF-induced neoplastic transformation in JB6 cells is related to blocking of Akt activation, and also attenuation of AP-1 activation for ellagitannins.

Keywords: *Elaeagnatus umbellata*, Elaeagnaceae; *Oenothera erythrosepala*, Onagraceae; *Geranium thubergii*, Geraniaceae; *Kunzea ambigua*, Myrtaceae; *Eucalyptus alba*, Myrtacaeae; Tannins; Cell transformation; AP-1; PI3K; Akt; MAP kinase; Ellagitannins; Ascorgeraniin; Oenothein B; Alienanin B; Pterocarinin A; Chromone gallates; Kunzeachromone A; Kunzeachromone D; Kunzeachromone F; Triterpenoid; Betulinic acid; Glycyrrhizin

1. Introduction

Cancer chemoprevention, which can be defined as the use of substances to prevent the process of cancer development (carcinogenesis), is one of the major strategies for cancer control. Natural components (phytochemicals) from numerous plants, to halt or retard the carcinogenic process, have been searched extensively in recent years.

Abbreviations: AP-1, activator protein 1; BME, basal medium Eagle; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EBV-EA, Epsten–Barr virus early antigen; EGCG, (−)-epigallochatechin-3-gallate; EGF, epidermal growth factor; EGFR, EGF receptor; ERKs, extracellular-signal regulated kinases; FBS, fetal bovine serum; JB6 P+, JB6 promotion sensitive cell line; JB6 P−, JB6 promotion resistant cell line; JNKs, c-JUN N-terminal kinases; MAP, mitogen-activated protein; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

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Tannins make up a large group of polyphenols found very widely throughout the plant kingdom and many exhibit anti-carcinogenic activities (Nepka et al., 1999). On the other hand, triterpenoids, a group of the major phytochemicals, have been also reported to have anti-carcinogenic effects (Tanaka et al., 2003; Konoshima et al., 1996). However, the mechanisms by which these phytochemicals display anti-carcinogenic effects are not clear.

Many of the molecular alterations in the carcinogenic process are associated with intercellular signal-transduction pathways that regulate cell proliferation and differentiation (Gescher et al., 1998; Bode and Dong, 2000; Surh, 2003), and numerous signal-transduction pathways converge with activation of transcription factors, such as activator protein 1 (AP-1), which leads to the expression of a number of target-genes. The functional activation of AP-1 is suggested to be associated with preneoplastic to neoplastic transformations in cell-culture and animal models (Dong et al., 1994; Bernstein and Colburn, 1989; Huang et al., 1997).

The JB6 cell system of clonal genetic variants, which are promotion sensitive (P+) or promotion resistant (P−), allows the study of genetic susceptibility to transformation, promotion and progression at the molecular level. The JB6 P+, P− and transformed variants are a series of cell lines representing 'earlier-to-later' stages of preneoplastic-to-neoplastic progression (Dong et al., 1994; Bernstein and Colburn, 1989; Colburn et al., 1979; Sun et al., 1993). Dong et al. (1994) have indicated that the AP-1 plays a critical role in tumor promotion through comparison of the JB6 P+ and P− cells.

Accumulating evidence also suggests the importance of phosphatidylinositol 3-kinase (PI3K) signaling in carcinogenesis (Stambolic et al., 1999; Krasilnikov, 2000; Chang et al., 1997; Klippel et al., 1998; Shayesteh et al., 1999; Phillips et al., 1998). We also demonstrated the involvement of the PI3K pathway on epidermal growth factor. (EGF)-induced cell transformation in JB6 P+ cells (Nomura et al., 2003). We have previously shown that (−)-epigallochatechin-3-gallate (EGCG), which exhibits anti-tumor promotion effects in several experiments (Fujiki et al., 1992; Dong et al., 1997), inhibited ultraviolet (UV)-induced activation of AP-1 and PI3K in the JB6 P+ cells (Nomura et al., 2000, 2001). Therefore, some tannins and triterpenoids may also have anti-tumor promotion activities and inhibitory effects of AP-1 and/or PI3K signaling pathways. Specifically, in this study, we investigated the effects of four ellagitannins, compounds **1**, **2** (from *Elaeagnatus umbellata*) (Ito et al., 1999a,b), **3** (from *Oenothera* species) (Hatano et al., 1990) and **4** (from *Geranium thubergii*) (Okuda et al., 1986), three chromone gallates, compounds **5**, **6** and **7** (from *Kunzea ambigua*) (Ito et al., 2004), and two triterpenoids, compounds **8** (from *Eucalyptus alba*) (Yoshida et al., 1996) and **9**, on EGF-induced cell transformation and its related cell-signaling pathways, such as AP-1 and PI3K/Akt signaling, using the JB6 P+ cell line.

2. Results and discussion

2.1. Tannins inhibited EGF-induced cell transformation

The mouse epidermal JB6 cell system is a well developed model for studying tumor promotion. Therefore, we used the JB6 Cl 41 promotion sensitive (P+) cell line to test the anti-tumor promoting effect of tannins and triterpenoids (Fig. 1). EGF induced 1000–2000 transformed colonies in soft agar. We found that ellagitannins (compounds **1**, **2**, **3** and **4**) and chromone gallates (compounds **6** and **7**), but not compound **5**, blocked the anchorage-independent cell growth (cell transformation) induced by EGF in a concentration-dependent manner (Fig. 2(a) and (b)). In particular, the inhibition rates of C-glucosidic ellagitannins, compounds **1** and **2**, were much stronger than those of the other tannins. The inhibition of cell transformation by the tannins was not due to growth inhibition, because at the concentrations tested none of the tannins had an effect on cell proliferation (data not shown). These results supported our reports that the C-glucosidic ellagitannins (Ito et al., 1999a,b), including compound **1**, and C-glucosylchromones (Ito et al., 2004), which resemble structurally the kunzeachromones, exhibited significant inhibitory effects on Epsten-Barr virus early antigen (EBV-EA) activation induced by TPA. Furthermore, compound **1** also has an inhibitory effect on the two-stage mouse skin carcinogenesis model (Ito et al., 1999a,b). On the other hand, although many kinds of triterpenoids have been reported to have anti-tumor promotion effects (Tanaka et al., 2003; Konoshima et al., 1996), compounds **8** and **9** did not show inhibition of cell transformation in the JB6 Cl 41 cells (Fig. 2(a)). The reason is not clear, but it may be due to different experimental approaches.

2.2. Effects of tannins on tyrosine phosphorylation of EGFR by EGF

Liang et al. (1997) demonstrated that EGCG inhibited protein tyrosine phosphorylation of the EGF receptor (EGFR) through blocking binding of EGF and EGFR. In addition, several flavonoids attenuate the auto-phosphorylation of EGFR by EGF (Agullo et al., 1997). Then, the influence of tannins, which blocks cell transformation, on EGF-induced tyrosine phosphorylation of EGFR was assessed. As shown in Fig. 3, compound **3**, but not other tannins, inhibits tyrosine phosphorylation of EGFR by EGF; inhibition of EGFR phosphorylation by compound **3** might be involved in blocking of cell transformation.

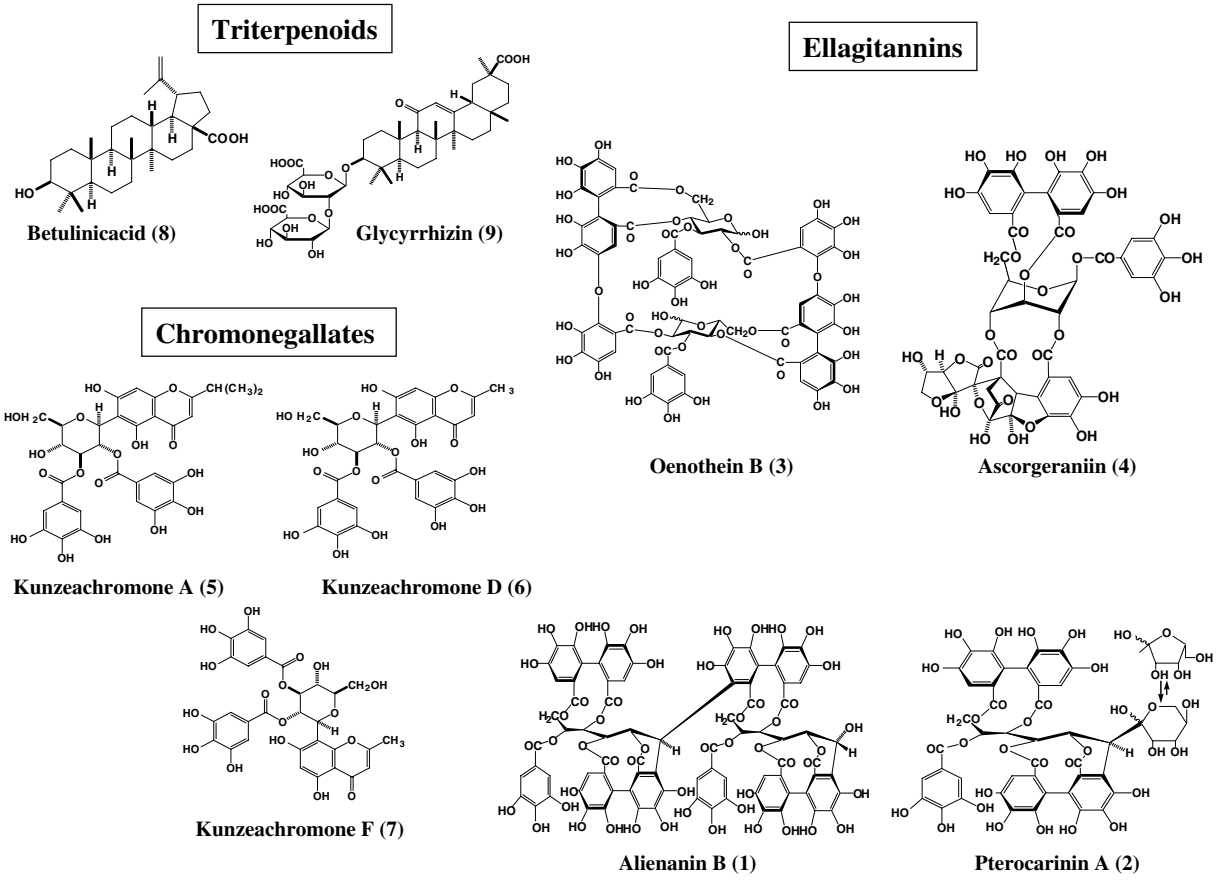

Fig. 1. Stuctures of tannins, chromone gallates and ellagitannins, and triterpenoids. Each compound number is indicated in parentheses.

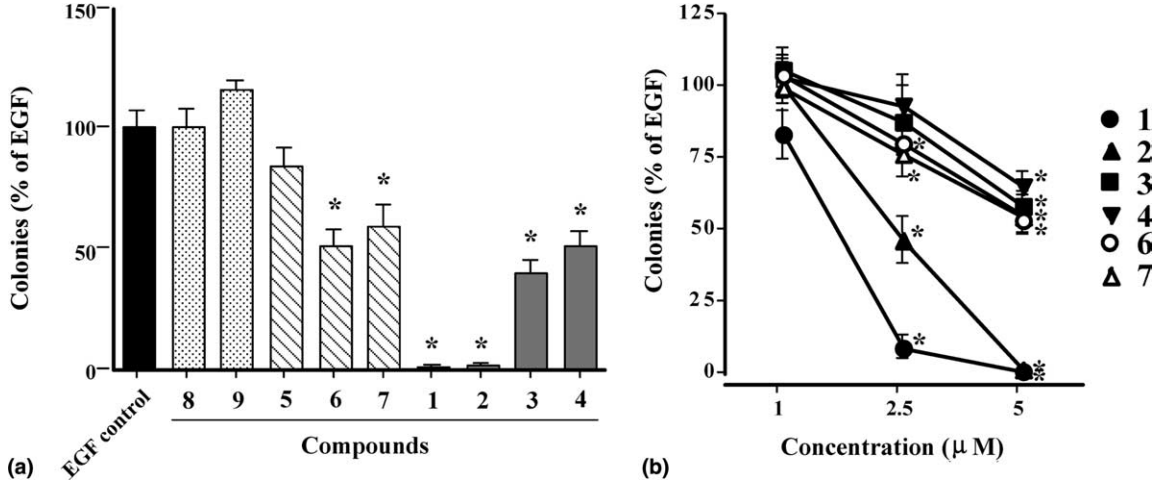

Fig. 2. Inhibition of EGF-induced cell transformation by tannins. Aliquots of 10^4 JB6 Cl 41 cells were treated with EGF (20 ng/ml) with or without 5 μM of tannins or triterpenoids (a) and different concentrations (b) of tannins in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after 14 days incubation at 37 °C in a 5% CO_2 incubator. Data are expressed as means ± S.D. of three experiments. *Significantly different from EGF alone at $P < 0.01$.

2.3. Ellagitannins attenuated EGF-induced AP-1 activation

The transcription factor, AP-1, plays a critical role in neoplastic transformation as well as in tumor promotion (Dong et al., 1994; Angel and Karin, 1991). Blocking of the tumor promoter-induced AP-1 activation inhibited neoplastic transformation in the JB6 cells (Dong et al., 1994; Huang et al., 1997). We therefore investigated whether inhibition of cell transformation by tannins is

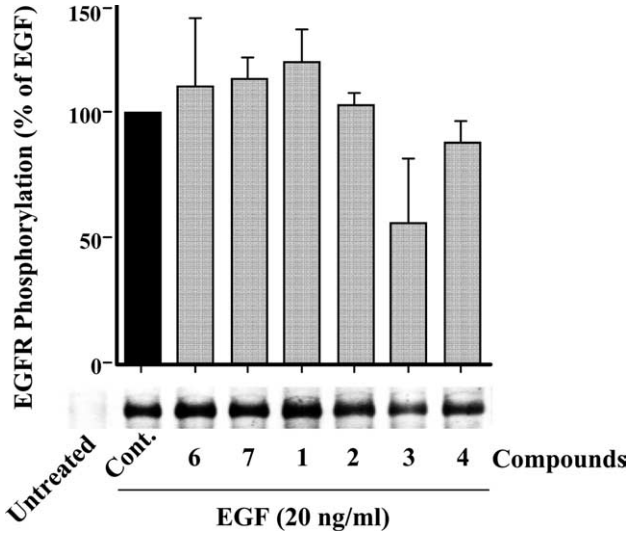

Fig. 3. Effect of tannins on EGF-induced EGFR phosphorylation. JB6 Cl 41 cells were pretreated with each tannin (5 μM) for 1 h. The cells were then treated or untreated with EGF (20 ng/ml) and subsequently incubated for 15 min. The phosphrylation levels of EGFR were estimated by immunoblotting as described in Section 3. Data are expressed as means ± S.D. of three experiments.

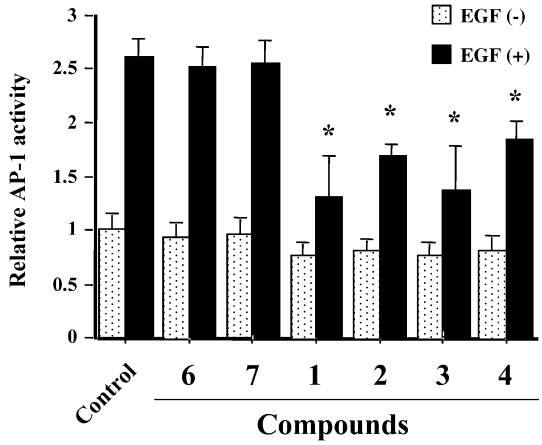

Fig. 4. Effect of tannins on EGF-induced AP-1 activation. JB6 AP-1 reporter stable P + 1–1 cells were exposed to 20 ng/ml EGF with or without each tannin (5 μM) for 24 h. The results are shown relative AP-1 activity expressed as means ± S.D. of three experiments. *Significantly different from EGF alone at $P < 0.05$.

involved in AP-1 activation. Ellagitannins (compounds **1**, **2**, **3** and **4**), but not chromone gallates (compounds **6** and **7**), significantly attenuated EGF-induced AP-1 activation (Fig. 4), suggesting that blocking of cell transformations by ellagitannins is, in part, due to attenuation of AP-1 activation induced by EGF. However, the structure–activity relationship of the ellagitannins is unclear.

2.4. Compounds *1* and *3* inhibited EGF-induced phosphorylation of ERKs and p38 kinases

AP-1 is a well-characterized transcription factor composed of either homo- or heterodimers between members of the JUN and FOS families (Angel and Karin, 1991) and is regulated by the mitogen-activated protein (MAP) kinase-signaling cascade (Kallunki et al., 1994; Huang et al., 1998; Watts et al., 1998; Kyriakis et al., 2004). Three classes of MAP kinases are known including extracellular-signal regulated kinases (ERKs), c-JUN N-terminal kinases (JNKs) and p38 kinases (Boulton et al., 1991; Kyriakis et al., 1994; Kyosseva, 2004; Rubinfeld and Seger, 2004). ERKs are activated by signals initiated by growth factors, including EGF (Boulton et al., 1991; Rubinfeld and Seger, 2004). Watts et al. (1998) have shown that ERKs have a critical role in AP-1 transactivation and neoplastic transformation induced by TPA and EGF in JB6 cells. In contrast, JNKs and p38 kinases are generally activated by various forms of stress (Kyriakis et al., 2004; Davis, 1994). However, JNKs and p38 kinase may also play a key role in signaling by growth factors (Minden et al., 1994; Rice et al., 2002). He et al. (2003) reported that p38 kinases regulate EGF-induced cell transformation through activation of its target factors, such as signal transducer and activator of transcription 1 (STAT1), c-Myc, and AP-1, in JB6 cells. In that paper, they also indicated that JNKs are not phosphorylated by EGF in JB6 cells. We then studied effects of tannins on EGF-induced phosphorylation of ERKs and p38 kinases and the results are shown in Fig. 5. Compounds **1** and **3** inhibited phosphorylation of ERKs and p38 kinases induced by EGF, while inhibition by other tannins, including compounds **2** and **4**, was not observed. These results suggest that compounds **1** and **3** attenuate EGF-induced AP-1 activation through inhibition of ERKs and p38 kinases, whereas attenuation of AP-1 activation by compounds **2** and **4** must involve unknown mechanisms.

2.5. Compounds *3* and *4* inhibited EGF-induced PI3K activation

PI3K is central to the co-ordinated control of multiple cell-signaling pathways leading to cell growth, proliferation, survival and migration (Krasilnikov, 2000; Wymann et al., 2003). Accumulating evidence suggests the importance of the PI3K-signaling pathway in carcinogenesis (Stambolic et al., 1999; Krasilnikov, 2000). We also have demonstrated that the PI3K-signaling pathway is closely related to EGF-induced cell transformation in JB6 cells (Nomura et al., 2003). In addition, we previously demonstrated that EGCG inhibited UV-induced PI3K activation (Nomura et al., 2001). We assessed the effects of the tannins on EGF-induced PI3K activation. The result revealed that compounds **3** and **4**, but not other tannins, suppressed EGF-induced PI3K activation (Fig. 6(a)).

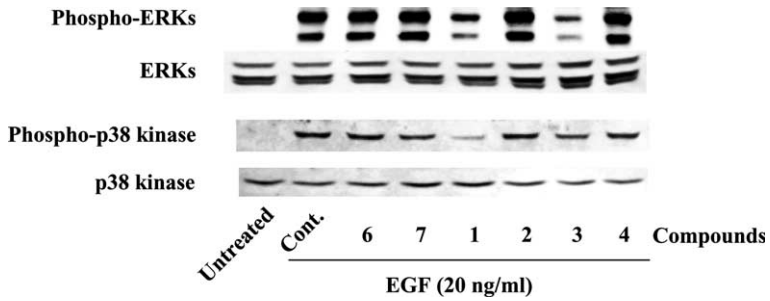

Fig. 5. Effect of tannins on EGF-induced phosphorylation of ERKs and p38 kinases. JB6 Cl 41 cells were pretreated with each tannin (5 μM) for 1 h. The cells were then treated or untreated with EGF (20 ng/ml) and subsequently cultured for 15 min. The phosphorylation levels were estimated by immunoblotting as described in Section 3.

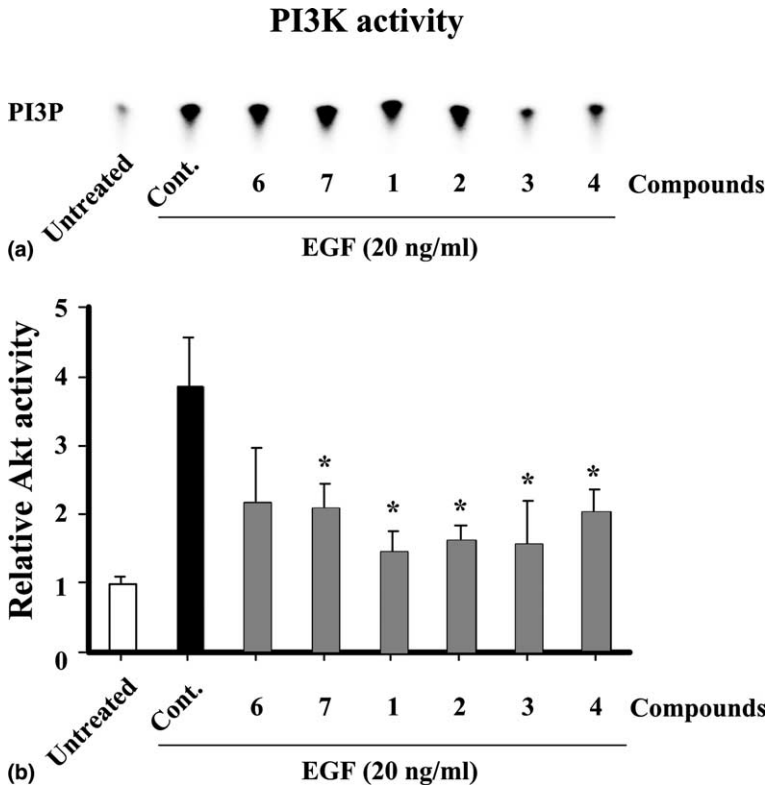

Fig. 6. Effect of tannins on EGF-induced activation of PI3K and Akt. JB6 Cl 41 cells were pretreated with each tannin (5 μM) for 1 h. The cells were then treated or untreated with EGF (20 ng/ml) and subsequently incubated for 15 min. (a) PI3K activities were determined as described in Section 3. PI3P, phosphoinositol 3-monophosphate. (b) The respective activities of Akt were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as means ± S.D. of three experiments. *Significantly different from the EGF alone at $P < 0.05$.

2.6. Tannins inhibited EGF-induced Akt activation

Akt, a downstream effector of PI3K, plays a central role in the PI3K-signaling pathway (Brazil et al., 2004). We have shown that overexpression of a dominant negative mutant of Akt1, which antagonizes Akt function, blocked EGF-induced cell transformation in the JB6 cells (Nomura et al., 2003). Therefore, we investigated the effects of the tannins on EGF-induced Akt activation. As shown in Fig. 6(b), all tannins that block cell transformation markedly inhibit EGF-induced Akt activation. This result suggests that inhibition of Akt activation by tannins is closely involved in blocking cell transformation. The mechanisms by which tannins, except compounds **3** and **4**, inhibit EGF-induced Akt activation are unclear at present, and will be further investigated to clarify the mechanisms involved.

2.7. Conclusion

Signal-transduction is referred to as cellular responses to signals from extracellular stimuli via a complicated

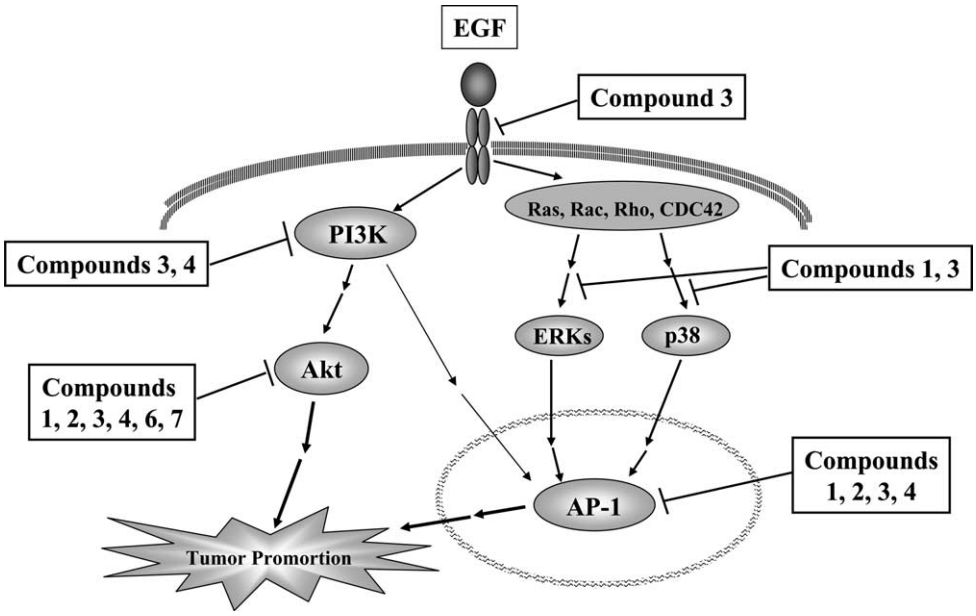

Fig. 7. Possible targets of the tannins in EGF-induced signal transduction pathways. Inhibition by the tannins is indicated by '⊥', extending from the box to the point of inhibition.

network of highly regulated events. Signal-transduction pathways, including AP-1 and PI3K pathways, have been focused as prime targets for chemopreventive phytochemicals (Surh, 2003). Our results suggest that inhibition by tannins of EGF-induced neoplastic transformation in JB6 cells is related to blocking of Akt activation, and also to attenuation of AP-1 activation for ellagitannins (Fig. 7). Although we could not completely explain the inhibition of cell transformation by the tannins from the results of the present study, our results provide additional insight into the biological actions of ellagitannins and chromone gallates and the molecular basis for the development of new chemopreventive agents for cancer.

3. Experimental

3.1. Materials

Ellagitannins, alienanin B (**1**), pterocarinin A (**2**) (from *E. umbellata*) (Ito et al., 1999a,b), oenothein B (**3**) (from *Oenothera* species) (Hatano et al., 1990) and ascorgeraniin (**4**) (from *G. thubergii*) (Okuda et al., 1986), chromone gallates, kunzeachromone A (**5**), D (**6**) and F (**7**) (from *K. ambigua*) (Ito et al., 2004), and a triterpenoid, betulinic acid (**8**) (from *E. alba*) (Yoshida et al., 1996), were isolated at the Faculty of Pharmaceutical Sciences, Okayama University (Okayama, Japan). Glycyrrhizin (**9**) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The structures are shown in Fig. 1. Eagle's minimum essential medium (MEM), L-glutamine and basal medium Eagle (BME) were from Life Technologies (Rockville, MD, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphatidylinositol and gentamicin were from Sigma (St. Louis, MO, USA); the Akt immunoprecipitation kinase assay kit was from Upstate Biotechnology Inc. (Lake Placid, NY, USA); the PhosphoPlus p44/42 MAP and p38 kinase antibody kits were from Cell Signaling Technology Inc. (Beverly, MA, USA); the anti-Akt1/2 antibody, anti-EGF receptor antibody, anti-phosphotyrosine antibody (PY20), agarose conjugated anti-phosphotyrosine antibody (PY99) and protein A/G plus-agarose were from Santa Cruz (Santa Cruz, CA, USA); dimethyl sulfoxide (DMSO) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

3.2. Cell cultures

The JB6 mouse epidermal cell line Cl 41 and its stable transfectant, P + 1–1 (AP-1 reporter transfectant), were gifts from Dr. Zigang Dong of the Hormel Institute, University of Minnesota, (Austin, MN, USA) and cultured at 37 °C in MEM supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin.

3.3. Anchorage-independent growth assay

JB6 Cl 41 cells (1×10^4) were exposed to EGF (20 ng/ml) with or without the indicated concentrations of tannins and triterpenoids in 1 ml 0.33% BME agar containing 10% FBS over 3.5 ml 0.5% BME agar medium containing 10% FBS. The cultures were maintained in

a 37 °C, 5% CO_2 incubator and the cell colonies were scored at 14 days after the cells were exposed to EGF.

3.4. Cell growth inhibition assay

JB6 Cl 41 cells (5×10^4) were seeded in each well of 96-well plates and allowed to attach overnight. They were then treated with the indicated concentration of the tannins and the triterpenoids for 72 h. Subsequently, MTT (25 μl, 2 mg/ml: Sigma) in phosphate-buffered saline (PBS) was added to each well, followed by incubation for 4 h at 37 °C. Formazan crystals were dissolved in DMSO. Absorbance was determined with a microplate reader (Multiskan BICHROMATIC, Labsystems Japan, Tokyo, Japan) at 540 nm.

3.5. EGFR phosphorylation assay

JB6 Cl 41 cells were cultured to 80% confluence and starved by culturing in 0.1% FBS/MEM for 24 h at 37 °C. The media were then changed to fresh 0.1% FBS/MEM and cells were incubated for another 2–4 h at 37 °C. Before the cells were exposed to EGF, they were either treated or untreated (control) with each tannin (5 μM) for 1 h. Then, EGF (20 ng/ml) was added and cells were subsequently incubated for an additional 15 min at 37 °C, then lysed in of lysis buffer (400 μl) [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin], and the lysate was sonicated and centrifuged. The quantity of protein in the supernatant fraction was normalized against the untreated control, and immunoprecipitation was performed using 20 μl of anti-EGF receptor (EGFR) antibody. The enzyme immune complex was washed three times with 0.5 ml of lysis buffer and twice with PBS, following which was added 100 μl of 1 × SDS sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), 0.01% bromophenol blue]. The immunoblot analysis was performed by using an anti-phosphotyrosine antibody (PY20). Antibody-bound proteins were detected by luminescence (ECL Western Blotting Kit, Amersham Biosciences Corp., Piscataway, NJ, USA) and analyzed using Typhoon 9400 (Amersham Biosciences).

3.6. Assay of AP-1 activity

JB6 AP-1 reporter stable P + 1–1 cells (7×10^3) were seeded in a 96-well plate. After 24 h incubation, the cells were starved by replacing 0.1% FBS/MEM for 24 h. Then, the cells were pretreated with each tannin (5 μM) for 1 h and cultured with 20 ng/ml EGF in the presence or absence of the tannins for 24 h. The cells were extracted with lysis buffer and luciferase activity was measured with the BioOrbit 1253 luminometer (Turku, Finland). Relative AP-1 activity was calculated as described previously (Nomura et al., 2000, 2003).

3.7. Immunoblotting

Immunoblotting was carried out as described previously (Nomura et al., 2000, 2003). In brief, JB6 Cl 41 cells were treated as described in "*EGFR phosphorylation assay*". The cells were then lysed, and immunoblot analysis performed by using the antibodies against p44/42 MAP kinases (ERKs) and p38 kinase antibody or the phospho-specific antibodies against their phosphorylated proteins. Antibody-bound proteins were detected by luminescence (ECL Western Blotting Kit, Amersham Biosciences) and analyzed using Typhoon 9400 (Amersham Biosciences).

3.8. PI3K assay

JB6 Cl 41 cells pretreated with each tannin (5 μM) for 1 h were exposed to EGF (20 ng/ml) for 15 min. The cells were lysed in 400 μl of lysis buffer [20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM aprotinin, 10 μM leupeptin], and the lysate was sonicated and centrifuged. The quantity of protein in the supernatant fraction was normalized against the untreated control, and incubated, with gentle rocking for 2 h at 4 °C, with agarose (20 μl) conjugated with a monoclonal anti-phosphotyrosine antibody (PY99). Then, PI3K activity was determined as described previously (Nomura et al., 2001). The radiolabeled spot of phosphatidylinositol 3-monophosphate (PI3P), that indicates PI3K activation, was quantified using a Bas-2000 bio-imaging analyzer (Fuji Film).

3.9. Akt immunoprecipitation kinase assay

JB6 Cl 41 cells were treated or untreated with each tannin (5 μM) before treatment with EGF (20 ng/ml) and lysates were prepared from the cells and the immunoprecipitation was carried out using 20 μl of anti-Akt1/2 antibody. Akt activities were determined using a specific Akt substrate peptide and γ-^{32}P ATP as described previously (Nomura et al., 2001).

3.10. Statistical analysis

Significant differences in this study were determined by using the Student's *t* test.

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