Raf and MEK Protein Kinases Are Direct Molecular Targets for the Chemopreventive Effect of Quercetin, a Major Flavonol in Red Wine

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Abstract

Considerable attention has focused on the health-promoting effects of red wine and its nonflavonoid polyphenol compound resveratrol. However, the underlying molecular mechanisms and molecular target(s) of red wine or other potentially active ingredients in red wine remain unknown. Here, we report that red wine extract (RWE) or the red wine flavonoid quercetin inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation of JB6 promotion-sensitive mouse skin epidermal (JB6 P+) cells. The activation of activator protein-1 and nuclear factor-κB induced by TPA was dose dependently inhibited by RWE or quercetin treatment. Western blot and kinase assay data revealed that RWE or quercetin inhibited mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 and Raf1 kinase activities and subsequently attenuated TPA-induced phosphorylation of ERK/p90 ribosomal S6 kinase. Although either RWE or quercetin suppressed Raf1 kinase activity, they were more effective in inhibiting MEK1 activity. Importantly, quercetin exerted stronger inhibitory effects than PD98059, a well-known pharmacologic inhibitor of MEK. Resveratrol did not affect either MEK1 or Raf1 kinase activity. Pull-down assays revealed that RWE or quercetin (but not resveratrol) bound with either MEK1 or Raf1. RWE or quercetin also dose dependently suppressed JB6 P+ cell transformation induced by epidermal growth factor or H-Ras, both of which are involved in the activation of MEK/ERK signaling. Docking data suggested that quercetin, but not resveratrol, formed a hydrogen bond with the backbone amide group of Ser212, which is the key interaction for stabilizing the inactive conformation of the activation loop of MEK1. [Cancer Res 2008;68(3):946–55]

Introduction

Considerable attention has been focused on the health benefits of red wine, which are associated with its high content of a variety of polyphenols (1). Red wine can also reportedly prevent various types of cancers (2). Although red wine contains different types of polyphenols, multiple lines of evidence indicate the health-promoting effects of resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is a nonflavonoid compound present in red wine. Previous studies reported that resveratrol exerted antitumor effects through the activation of p53-mediated apoptosis (3) and inhibited cyclooxygenase-2 expression and two-stage skin cancer induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin (4). However, the concentration of resveratrol required for achieving its beneficial effects is unlikely to be attained through a moderate consumption of red wine due to its low content of resveratrol. The resveratrol content in red wine (0.6–6.8 mg/L in French red wines (5)) is ~30 times lower than the flavonoid content, with one of the major flavonols in red wine being quercetin (3,3',4',5'-pentahydroxyflavone; ref. 6). Quercetin has been suggested as a potent anticarcinogenic flavonol. In 9,10-dimethyl-1,2-benzanthracene-initiated and TPA-promoted two-stage mouse skin cancer models, quercetin exerted the strongest anticarcinogenic effects (7). Thus, the identification of the active actual components responsible for the chemopreventive effects of red wine and molecular mechanism(s) of action is needed.

Activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) act as pivotal transcription factors involving neoplastic transformation and development of cancer (8–11) and are regulated by upstream kinases, including mitogen-activated protein kinase (MAPK) signaling pathways. MAPK signaling pathways are commonly up-regulated in various cancer cell types, and these pathways are involved in cell proliferation and survival (12). Among the components of the MAPK pathways, the MAPK kinase kinase (Raf)/MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK cascade has been the focus of cancer chemotherapy because of its relevance in carcinogenesis. A variety of tumor promoters, including TPA and epidermal growth factor (EGF), are known to induce neoplastic transformation through activation of the Raf/MEK/ERK pathway in various cell lines (8, 13, 14). The Raf/MEK/ERK pathway has also been identified as a key downstream effector of Ras, which is a frequently mutated oncoprotein in 30% or more of human cancers (15, 16). This pathway plays a critical role in linking extracellular signals associated with Ras activation to nuclear transcription events (17). Because aberrant activation of ERK has been shown in various types of tumors (18, 19), the targeted down-regulation of ERK through the inhibition of upstream kinases, such as Raf or MEK, is an effective method for intervening in carcinogenesis.

The JB6 mouse epidermal cell system, including promotion-sensitive (P+) and promotion-resistant (P–) components, is regarded as an appropriate model for studying tumor promoter–induced carcinogenic processes at the molecular level. The present

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study aimed to elucidate the mechanism of the antitumorigenic effects of red wine and to identify potentially effective compounds by investigating the possible inhibitory effects of red wine extract (RWE), quercetin, or resveratrol on TPA-induced neoplastic transformation of JB6 P+ cells. Here, we report that RWE or quercetin, but not resveratrol, is a potent inhibitor of MEK1 activity. The inhibition of MEK1 suppressed downstream ERK phosphorylation and activation of AP-1 and NF-κB, which subsequently inhibited neoplastic transformation. RWE or quercetin also inhibited Raf1 activity, but this was less substantial than the inhibition of MEK1.

Materials and Methods

Chemicals. Quercetin, resveratrol, EGF, and TPA were obtained from Sigma Chemical; Eagle’s MEM, basal medium Eagle (BME), gentamicin, and t-glutamine were purchased from Life Technologies; and fetal bovine serum (FBS) was purchased from Gemini Bio-Products. PD98059 and GW5074 were obtained from Calbiochem. The antibodies against phosphorylated MEK (Ser217/218), phosphorylated ERK (Thr202/Tyr204), total ERK, phosphorylated p90 ribosomal S6 kinase (p90RSK; Thr359/Ser363), p90RSK, phosphorylated c-Jun N-terminal kinase (JNK; Thr183/Tyr185), and total JNK were purchased from Cell Signaling Technology. The antibodies against total MEK1 and Raf1 were from Santa Cruz Biotechnology. The MEK1 and Raf1 kinase assay kits were obtained from Upstate Biotechnology. CNBr-nephrotoxic snake venom detection kit were purchased from Amersham Pharmacia Biotech, and the luciferase assay substrate were purchased from Promega. The luciferase activity was measured using a luminometer (Luminoskan Ascent, Thermo Electron).

Western blot analysis. After the cells (1.5 × 10^5) were cultured in a 10-cm dish for 48 h, they were starved in serum-free medium for an additional 24 h. The cells were then treated with RWE (0-20 μg/mL) or quercetin (0-20 μg/mL) for 1 h before they were exposed to 20 ng/mL TPA for an additional 30 min. The harvested cells were disrupted and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit as described in the manufacturer’s manual. Lysate protein (20 μg) was subjected to 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. After blotting, the membrane was incubated with the specific primary antibody at 4°C overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with a horseradish peroxidase–conjugated secondary antibody.

In vitro MEK1 and Raf assays. The in vitro kinase assays were performed in accordance with the instructions provided by Upstate Biotechnology. In brief, every reaction contained 20 μL of assay dilution buffer [20 mmol/L MOPS (pH 7.2), 25 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT] and a magnesium-ATP cocktail buffer. For MEK1, 1 μg of the inactive ERK2 substrate peptide was also included. For Raf1, 0.4 μg of inactive MEK1 and 1 μg of inactive ERK2 were included. A 4-μL aliquot was removed from the reaction mixture, containing 20 μg of MBP substrate peptide and 10 μL of diluted [γ-32P]ATP solution, and incubated at 30°C for 30 min. This mixture was incubated for 10 min at 30°C, and then 25 μL aliquots were transferred onto p81 filter paper and washed thrice with 0.75% phosphoric acid for 5 min per wash and once with acetone for 2 min. The radioactivity incorporation was determined using a scintillation counter (LS6500; Beckman Coulter). Each experiment was performed thrice.

Ex vivo MEK1 and Raf1 immunoprecipitation and kinase assay. JB6 P+ cells were cultured to 80% confluence and then serum starved in 0.1% FBS/MEM for 24 h at 37°C. Cells were either treated or not treated with RWE, quercetin, or resveratrol for 1 h and then treated with 20 ng/mL TPA for 30 min, disrupted with lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L β-glycerophosphate, 1 mg/mL leupeptin, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)], and finally centrifuged at 14,000 rpm for 10 min in a microcentrifuge. The lysates, each containing 500 μg of protein, were used for immunoprecipitation with an antibody against MEK1 or Raf1 and then incubated at 4°C overnight. Protein A/G Plus agarose beads were then added and the mixture was continuously rotated for an additional 3 h at 4°C. The beads were washed thrice with kinase buffer [20 mmol/L MOPS (pH 7.2), 25 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT] and then resuspended in 20 μL of 1× kinase buffer supplemented with 1 μg of inactive ERK2 (for MEK1) or with 0.4 μg of inactive MEK1 and 1 μg of inactive ERK2 (for Raf1) and incubated for an additional 30 min at 30°C. 10 μL of diluted [γ-32P]ATP solution were added and mixed the mixture was incubated for 10 min at 30°C. A 20-μL aliquot was transferred onto p81 filter paper and washed thrice with 0.75% phosphoric acid for 5 min per wash and once with acetone for 2 min. The radioactivity incorporation was determined using a scintillation counter. Each experiment was performed thrice.

In vitro and ex vivo pull-down assays. Recombinant MEK1 (2 μg or Raf1) or a JB6 P+ cellular supernatant fraction (500 μg protein) was incubated with the RWE-Sepharose 4B or quercetin-Sepharose 4B (or Sepharose 4B as control) beads (100 μL, 50% slurry) in reaction buffer [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 2 μg/mL bovine serum albumin, 0.02 mmol/L PMSF, 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with buffer [50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP40, 0.02 mmol/L PMSF], and proteins bound to the beads were analyzed by immunoblotting.
**Results**

**RWE inhibits TPA-induced neoplastic transformation of JB6 P+ cells.** To investigate whether red wine exerts health-promoting effects by intervening in carcinogenesis processes, we first examined the effect of RWE on neoplastic transformation. Results indicated that treatment with RWE markedly inhibited TPA-promoted neoplastic transformation of JB6 P+ cells in a dose-dependent manner (Fig. 1A). Based on the numbers of cell colonies, RWE at only 5 μg/mL suppressed TPA-induced JB6 P+ cell transformation by 59% and, at or above 20 μg/mL, almost completely prevented transformation (Fig. 1B).

**RWE inhibits TPA-induced AP-1 and NF-κB transactivation in JB6 P+ cells.** AP-1 and NF-κB are major transcription factors involved in TPA-induced neoplastic transformation of JB6 P+ cells (22–24). To investigate whether RWE down-regulates cell transformation through the inhibition of these transcription factors, we measured AP-1 and NF-κB transactivation by using JB6 P+ cells stably transfected with an AP-1 or NF-κB luciferase reporter plasmid. RWE inhibited TPA-induced transactivation of either AP-1 or NF-κB in a dose-dependent manner, and treatment with RWE at a low concentration inhibited AP-1 more effectively compared with NF-κB (Fig. 1C and D, respectively).

**RWE suppresses TPA-induced phosphorylation of MEK, ERK, and p90RSK in JB6 P+ cells.** Among the kinases belonging to the MAPK family, ERK has been reported to be the most important in TPA-induced JB6 P+ cell transformation (9, 24, 25). We next examined whether RWE down-regulates ERK and JNK pathways stimulated by TPA in JB6 P+ cells. RWE at 5 μg/mL inhibited TPA-induced phosphorylation of MEK but more strongly suppressed phosphorylation of the MEK downstream kinases ERK and p90RSK (Fig. 1A). RWE, at 20 μg/mL, slightly inhibited TPA-induced JNK phosphorylation but to a much lower degree than ERK (data not shown). These results suggested that the inhibition of the MEK/ERK/p90RSK signal pathway by RWE leads to the suppression of AP-1 and NF-κB, resulting in decreased neoplastic transformation.

**RWE inhibits MEK1 activity more strongly than Raf1 activity.** We next investigated the effects of RWE on the kinase activity of MEK1 and Raf1. Kinase assay data revealed that RWE inhibited MEK1 activity more strongly than Raf1 activity (Fig. 2A). RWE at 0.5 μg/mL blocked active MEK1 activity by 53.2%, whereas it had no effect on active Raf1 activity. In vitro MEK1 activity was completely inhibited by RWE at or above 5 μg/mL, whereas treatment with 5 μg/mL RWE reduced Raf1 activity by only 24.9%.

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**Molecular modeling.** Insight II (Accelrys) was used for the docking study and structure analysis with the crystal coordinates of MEK1 (accession code 1S9J), which are available in the Protein Data Bank.6

**Statistical analysis.** When necessary, data were expressed as means ± SD values, and the ANOVA was used for multiple statistical comparisons. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

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6 http://www.rcsb.org/pdb/
Consistent with results from an in vitro kinase assay, an ex vivo kinase assay also revealed that RWE inhibited TPA-induced MEK1 activity in JB6 P+ cells more than Raf1 activity. RWE at 1 μg/mL blocked TPA-induced MEK1 activity by ~34.6%, whereas no significant inhibition against TPA-induced Raf1 activity was detected. However, at the highest concentration, RWE effectively suppressed either MEK1 or Raf1 activity stimulated by TPA (Fig. 2B, bottom). These results indicated that the inhibition of
cell transformation by RWE was mainly caused by the suppression of MEK1 activity and to a lesser extent by inhibition of Raf1 and its downstream signaling pathways.

**RWE directly binds with either MEK1 or Raf1.** To determine whether the inhibition of MEK1 and Raf1 kinase activities by RWE was caused by a direct interaction, we next performed pull-down assays. Using an in vitro pull-down assay, MEK1 was found in the RWE-Sepharose 4B beads (Fig. 2C, left) but not in Sepharose 4B beads alone (Fig. 2C, left). We also observed ex vivo binding between RWE and MEK1 in JB6 P+ cell lysates (Fig. 2C, right). Because RWE could also inhibit Raf1 activity, we performed in vitro pull-down assays to determine whether RWE directly interacts with Raf1. Raf1 was found in the RWE-Sepharose 4B beads (Fig. 2D, left) but not in Sepharose 4B beads alone (Fig. 2D, left). As for MEK, we also observed ex vivo binding of RWE and Raf1 in JB6 P+ cell lysates (Fig. 2D, right). These results suggested that the inhibition of MEK1 or Raf1 activities by RWE occurs through direct binding of RWE with either protein.

**Quercetin inhibits MEK1 activity more strongly than Raf1 activity, whereas resveratrol has no inhibitory effect on either kinase.** To elucidate the active components of RWE contributing to its chemopreventive effects, we next examined the effects of quercetin or resveratrol (Fig. 3A) on MEK1 and Raf1 kinase activities. Similar to RWE, quercetin inhibited MEK1 activity more effectively compared with inhibition of Raf1 activity, whereas resveratrol had no inhibitory effect on either kinase (Fig. 3B). MEK1 kinase assay revealed that quercetin at 5 μM almost completely blocked MEK1 activity, and the effect was greater than the inhibition by PD098059, a specific MEK inhibitor. Furthermore, resveratrol at up to 20 μM exerted no effect on MEK1 activity (Fig. 3B, left). In contrast, quercetin inhibited Raf1 activity by only 23.8% at 10 μM, and resveratrol at up to 10 μM exerted no
effect (Fig. 3b, right). GW5074, a well-known inhibitor of Raf1, was used as a positive control.

To further determine whether quercetin directly binds with MEK1 or Raf1, we performed in vitro and ex vivo pull-down assays. MEK1 was found in the quercetin-Sepharose 4B beads but not in Sepharose 4B beads alone (Fig. 3C, top left). We also observed ex vivo binding of quercetin and MEK1 in JB6 P+ cell lysates (Fig. 3C, top right). In addition, Raf1 was found in the quercetin-Sepharose 4B beads but not in Sepharose 4B beads alone (Fig. 3C, bottom left). We also observed ex vivo binding of quercetin and Raf1 in JB6 P+ cell lysates (Fig. 3C, bottom right).

Western blot analysis confirmed that quercetin inhibited TPA-induced ERK phosphorylation, whereas quercetin had no effect on TPA-induced MEK phosphorylation in JB6 P+ cells (Fig. 3D). This is consistent with the previous result showing that quercetin blocked MEK1 activity more effectively than Raf1 activity. In addition, quercetin (at up to 20 μmol/L) had no effect on TPA-induced phosphorylation of JNK in JB6 P+ cells (data not shown). These results indicated that quercetin interacts with MEK1 and Raf1 protein kinases and results in down-regulation of their kinase activities. Of note, MEK is a more important target molecule of quercetin for the suppression of TPA-induced JB6 P+ cell transformation.

Quercetin inhibits TPA-induced neoplastic transformation and transactivation of AP-1 and NF-κB in JB6 P+ cells, whereas resveratrol had no effect. To confirm that inhibition of the Raf/MEK/ERK signaling pathway by quercetin leads to the suppression of neoplastic transformation, we next examined the effects of quercetin on TPA-induced JB6 P+ cell transformation and transactivation of AP-1 and NF-κB. Treatment with quercetin markedly inhibited TPA-promoted neoplastic transformation of JB6 P+ cells in a dose-dependent manner (Fig. 4A). Based on the numbers of cell colonies, quercetin at 40 μmol/L almost completely suppressed TPA-induced neoplastic transformation, whereas resveratrol at up to 40 μmol/L had no effect. To determine whether the blocking of transformation by quercetin involves the inhibition of AP-1 or NF-κB activity, we measured AP-1 and NF-κB transactivation. Quercetin suppressed TPA-induced transactivation of either AP-1 or NF-κB in a dose-dependent manner (Fig. 4B and C). Although resveratrol at 40 μmol/L slightly inhibited TPA-induced AP-1 or NF-κB activity, this inhibition might have been insufficient to suppress TPA-induced transformation. PD098059 and GW5074 were used as positive controls, and the inhibitory effect of quercetin was greater than that of PD098059 for treatment at the same concentration.

RWE or quercetin inhibits H-Ras–induced or EGF-induced neoplastic transformation of JB6 P+ cells, whereas resveratrol has no effect. Because H-Ras or EGF acts as a potent activator of the Raf/MEK/ERK signaling pathway leading to neoplastic transformation in JB6 P+ cells (9, 26, 27), we next examined whether RWE, quercetin, or resveratrol could inhibit H-Ras–induced or EGF-induced JB6 P+ cell transformation. RWE at 20 μg/mL suppressed H-Ras–induced or EGF-induced neoplastic transformation by 87.1% and 91.2%, respectively (Fig. 5A and B, respectively). Based on the number of cell colonies, quercetin at 20 μmol/L blocked H-Ras–induced or EGF-induced cell transformation by 94.7% or 66.31%, respectively (Fig. 5C and D, respectively), and this ability was significantly better than or similar to that of PD098059 or GW5074 at the same concentration, respectively. Consistent with other results, resveratrol at up to 20 μmol/L had no significant effect on H-Ras–induced or EGF-induced transformation. Because H-Ras or EGF acts as a potent activator of the Raf/MEK/ERK signaling pathway leading to neoplastic transformation in JB6 P+ cells (9, 26, 27), we next examined whether RWE, quercetin, or resveratrol could inhibit H-Ras–induced or EGF-induced JB6 P+ cell transformation. MEK1 was found in the quercetin-Sepharose 4B beads but not in Sepharose 4B beads alone (Fig. 3C, top left). We also observed ex vivo binding of quercetin and MEK1 in JB6 P+ cell lysates (Fig. 3C, top right). In addition, Raf1 was found in the quercetin-Sepharose 4B beads but not in Sepharose 4B beads alone (Fig. 3C, bottom left). We also observed ex vivo binding of quercetin and Raf1 in JB6 P+ cell lysates (Fig. 3C, bottom right). Western blot analysis confirmed that quercetin inhibited TPA-induced ERK phosphorylation, whereas quercetin had no effect on TPA-induced MEK phosphorylation in JB6 P+ cells (Fig. 3D). This is consistent with the previous result showing that quercetin blocked MEK1 activity more effectively than Raf1 activity. In addition, quercetin (at up to 20 μmol/L) had no effect on TPA-induced phosphorylation of JNK in JB6 P+ cells (data not shown). These results indicated that quercetin interacts with MEK1 and Raf1 protein kinases and results in down-regulation of their kinase activities. Of note, MEK is a more important target molecule of quercetin for the suppression of TPA-induced JB6 P+ cell transformation.

Quercetin inhibits TPA-induced neoplastic transformation and transactivation of AP-1 and NF-κB in JB6 P+ cells, whereas resveratrol had no effect. To confirm that inhibition of the Raf/MEK/ERK signaling pathway by quercetin leads to the suppression of neoplastic transformation, we next examined the effects of quercetin on TPA-induced JB6 P+ cell transformation and transactivation of AP-1 and NF-κB. Treatment with quercetin markedly inhibited TPA-promoted neoplastic transformation of JB6 P+ cells in a dose-dependent manner (Fig. 4A). Based on the numbers of cell colonies, quercetin at 40 μmol/L almost completely suppressed TPA-induced neoplastic transformation, whereas resveratrol at up to 40 μmol/L had no effect. To determine whether the blocking of transformation by quercetin involves the inhibition of AP-1 or NF-κB activity, we measured AP-1 and NF-κB transactivation. Quercetin suppressed TPA-induced transactivation of either AP-1 or NF-κB in a dose-dependent manner (Fig. 4B and C). Although resveratrol at 40 μmol/L slightly inhibited TPA-induced AP-1 or NF-κB activity, this inhibition might have been insufficient to suppress TPA-induced transformation. PD098059 and GW5074 were used as positive controls, and the inhibitory effect of quercetin was greater than that of PD098059 for treatment at the same concentration.

Figure 4. Comparison of inhibitory effects of quercetin or resveratrol against TPA-induced neoplastic transformation and AP-1 and NF-κB transactivation in JB6 P+ cells. A, quercetin was more effective than resveratrol at inhibiting TPA-induced JB6 P+ cell transformation. JB6 P+ cells were treated as described in Materials and Methods, and colonies were counted 14 d later under a microscope with the aid of Image-Pro Plus software (version 4). The effects of quercetin or resveratrol on neoplastic transformation of JB6 P+ cells are presented as the percent inhibition of cell transformation compared with cells treated with only TPA in soft agar. Columns, mean of percent inhibition as determined from three separate experiments; bars, SD. *, P < 0.05, significant difference between groups treated with TPA and quercetin (or resveratrol or PD098059 or GW5074) together and the group treated with TPA alone. B and C, TPA-induced activation of AP-1 (B) or NF-κB was inhibited more strongly by quercetin than by resveratrol. The JB6 P+ cells, which were stably transfected with AP-1 or NF-κB luciferase reporter plasmids, were pretreated with quercetin (or resveratrol or PD098059 or GW5074) for 1 h followed by exposure to 20 ng/mL TPA for 24 h. The relative activity was measured by the luciferase assay as described in Materials and Methods. Columns, mean of AP-1 and NF-κB luciferase activities calculated from three independent experiments; bars, SD. *, P < 0.05, significant differences between groups treated with TPA and quercetin (or resveratrol or PD098059 or GW5074) together and the group treated with TPA alone.
EGF-induced neoplastic transformation of JB6 P+ cells (Fig. 5C and D). Taken together, these findings support the idea that RWE or quercetin suppresses cell transformation mainly by targeting the Raf1/MEK/ERK signaling cascades.

Discussion

Although red wine has been reported to exert anticarcinogenic effects, including growth inhibition of some cancer cells (28, 29), the underlying mechanisms and molecular targets remain unclear. In the present study, we found that RWE inhibited TPA-induced neoplastic transformation of JB6 P+ cells. Previous studies have focused on the critical role of AP-1 in regulating transformation of JB6 P+ cells because blocking of AP-1 activity by phytochemicals is linked to the suppression of JB6 P+ cell transformation (11, 25, 30). However, other studies have shown that NF-κB activation is also required for TPA-induced neoplastic transformation of JB6 P+ cells.
Interestingly, a close interaction of c-Fos or c-Jun (AP-1 subunits) with p65 (a NF-κB subunit) has been reported (22). These findings suggest that inhibiting both or either transcription factor effectively suppresses neoplastic transformation. Our results showed that RWE inhibited TPA-induced activation of both AP-1 and NF-κB in JB6 P+ cells.

Transcription factors such as AP-1 and NF-κB are regulated mainly by MAPKs (31, 32). Previous studies have shown the importance of the involvement of ERK in JB6 P+ cell transformation. In contrast to P+ cells, P− cells do not respond to tumor promoters, and this was shown to be due to low levels of phosphorylated and total ERK proteins (9). The ERK signaling pathway involves Raf, MEK, ERK, and p90RSK proteins (33, 34). In this study, RWE inhibited TPA-induced phosphorylation of MEK, ERK, and p90RSK in JB6 P+ cells, and this inhibition of the ERK pathway led to the suppression of neoplastic transformation through the inhibition of AP-1 and NF-κB. RWE was more effective at inhibiting MEK1 activity than Raf1 activity, which is consistent with the above results. A strong inhibition of MEK1 activity resulted in RWE being very effective at suppressing phosphorylation of ERK due to this being a downstream effector kinase of MEK. In addition to the ERK pathway, JNK has been reported to involve AP-1 activation and neoplastic transformation in JB6 P+ cells (35). Other studies have found that JNK2-deficient mice failed to induce skin tumorigenesis in response to TPA, which also supports the important role of JNK in skin tumorigenesis (36). This prompted us to examine the effect of RWE on TPA-induced JNK phosphorylation in JB6 P+ cells. RWE at only 20 μmol/L slightly inhibited TPA-induced JNK phosphorylation. These results indicated that the inhibition of the ERK pathway by RWE might be responsible for the strong inhibition of neoplastic transformation of RWE. Further, in vitro and ex vivo pull-down assays revealed that RWE bound with either MEK1 or Raf1, which may contribute to the observed reduced kinase activities of MEK1 and Raf1.

The identification of the actual active components responsible for the chemopreventive effects of red wine and elucidation of the molecular mechanism(s) of action are needed. The present study compared the effects of resveratrol and quercetin and results indicated that quercetin inhibited MEK1 and Raf1 activities, whereas resveratrol at up to 20 μmol/L had no effect. Quercetin inhibited MEK1 activity more efficiently than Raf1 activity. Of note, quercetin was more effective than PD98059 for inhibiting MEK1 activity. Quercetin inhibited MEK1 and Raf1 activities through direct binding with each protein and blocking of MEK1 activity inhibited TPA-induced ERK phosphorylation in JB6 P+ cells. Interestingly, however, the TPA-induced MEK phosphorylation was not suppressed by quercetin, although quercetin slightly blocked Raf1 activity. These results suggested the presence of a negative feedback loop in MEK regulation. Other investigators have found that inhibition of MEK activity by PD184352 elevated MEK phosphorylation because the binding of PD184352 prevents the catalytic activity of MEK but still allows the phosphorylation of Ser218 and Ser222 (37). Therefore, because quercetin strongly inhibited MEK1 kinase activity, phosphorylation of MEK would not change in spite of the slight inhibition of Raf1 activity by quercetin. Quercetin, but not resveratrol, inhibited TPA-induced AP-1 and NF-κB activation by suppressing the MEK/ERK, but not the JNK, pathway, subsequently leading to the suppression of neoplastic transformation. Further investigation revealed that RWE or quercetin suppressed not only TPA-induced but also

![Figure 6. Modeling study of the MEK1 binding of quercetin, resveratrol, or kaempferol. A, hypothetical model of MEK1-quercetin complex. Quercetin (white) binds to the pocket adjacent to the ATP-binding (orange) site. PD318088 (green) is superimposed on the model structure of MEK1-quercetin complex for comparison. Yellow, partially disordered activation loop. The residues involved in the interactions with quercetin are indicated. Dashed lines, hydrogen bonds. B, hypothetical model of MEK1 in complex with resveratrol (green) or kaempferol (orange). Although each of these compounds can retain the hydrogen bond with Val127 and the van der Waals interactions involved in the binding of quercetin to MEK1, neither compound can form a hydrogen bond with the activation loop of MEK1 due to the lack of a hydrogen bond acceptor at the 3' position of their respective ring adjacent to the activation loop.](cancerres.aacrjournals.org/FIG6.jpg)
H-Ras–induced or EGF-induced neoplastic transformation, and these results support the finding that RWE or quercetin inhibits the MEK/ERK pathway regardless of the types of inducers stimulating this pathway.

In contrast to other protein kinase inhibitors, MEKI1 inhibitors, including PD098059, U0126, PD184352, and PD318088, do not compete with ATP, which accounts for their high selectivity (38). PD184352 is another MEKI1-specific inhibitor used in clinical trials, and it binds in a unique inhibitor-binding pocket that is adjacent to the ATP-binding site (39). In contrast to the highly homologous ATP-binding site, this unique binding pocket of MEK contains distinctive sequences that are not shared with other kinases. The binding of PD184352 with MEKI1 results in a stabilized inactive conformation and a deformation of catalytic sites (39).

In summary, RWE or quercetin inhibited tumor promoter–induced neoplastic transformation of JB6 P+ cells. This inhibition was mediated mainly through the blocking of the Raf/MEK/ERK/p90RSK pathway and subsequent suppression of AP-1 and NF-κB activity. RWE or quercetin binds with MEKI1 or Raf1 but inhibits MEKI1 activity more strongly than Raf1 activity. Together, these results suggested that MEKI1 is the most potent molecular target of RWE or quercetin for suppressing neoplastic transformation and that the chemopreventive effects of RWE are more likely to be attributable to quercetin than to resveratrol (Fig. 6B).

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References


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