Differential Effect of Retinoic Acid on Growth Regulation by Phorbol Ester in Human Cancer Cell Lines

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Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and all-trans-retinoic acid (trans-RA) are potent regulators of growth of cancer cells. In this study, we investigated the effect of TPA and trans-RA alone or their combination on proliferation of human breast cancer ZR75-1 and T47D and lung cancer H460 and H292 cell lines. trans-RA caused various degrees of growth inhibition of these cell lines. However, TPA showed inhibition of proliferation of H460 and H292 cells and induction of ZR75-1 cell growth. Although trans-RA did not significantly regulate the growth inhibitory effect of TPA, it completely prevented its growth stimulating function. The divergent effects of TPA were associated with specific disruption of cell cycle events, an induction of G0/G1 arrest in H460 and H292 cells and inhibition of G0/G1 arrest with increase of S phase in ZR75-1 cells. Induction of G0/G1 arrest was accompanied by induction of p21WAF1 and ERK activity, whereas inhibition of G0/G1 arrest was associated with enhanced activity of JNK and AP-1 but not ERK. trans-RA did not affect TPA-induced p21WAF1 expression. However, it inhibited TPA-induced AP-1 activity in ZR75-1 cells and the constitutive AP-1 activity in H460 and H292 cells. Thus, trans-RA modulates TPA activity through its interaction through TPA-induced JNK/AP-1 pathway but not TPA-induced ERK/p21WAF1 pathway.

Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent regulator of growth of many different cell types. It activates protein kinase C, which plays a key role in the control of many signal transduction pathways involved in different cellular functions, such as growth, differentiation, and cell transformation (2–4). Protein kinase C overexpression is associated with increased tumorigenicity and metastatic potential in several experimental models (5), and its activity is increased in tumors of breast and lung as compared with their normal counterparts (6). Activation of protein kinase C by TPA can lead to growth stimulation and cellular transformation (5–7) and is in part due to induction of AP-1, a collection of sequence-specific transcriptional activators composed of members of the c-Jun and c-Fos families, which are often associated with proliferation of cancer cells (8). TPA could also induce growth arrest and differentiation in certain leukemia cells and cancer cells, which is accompanied by induction of p21WAF1 (9–11). Recent studies have demonstrated that induction of p21WAF1 depends on Ras/ERK signaling and involves transcriptional activation of the p21WAF1 promoter in a p53-independent manner (12). P21WAF1 is believed to inhibit cell cycle progression through its interaction with cyclin-dependent kinase complexes, which are required for various cell cycle transitions (13, 14). Thus, TPA can either stimulate or inhibit cell proliferation, depending on cell type.

All-trans-retinoic acid (trans-RA) and its natural and synthetic derivatives (retinoids) regulate a broad range of biological processes, including growth, differentiation, and development in both normal and neoplastic cells (15, 16). The effect of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors and retinoids X receptors, that are encoded by three distinct genes, (α, β, and γ) and are members of the steroid/thyroid hormone receptor superfamily (17–19). Retinoid receptors modulate the expression of their target genes in response to their natural ligands trans-RA and 9-cis-RA by interacting as either homodimers or heterodimers with RA response elements. A number of RA target genes have been identified and many of them are associated with cell proliferation, differentiation, and growth (17–19).

In addition to transactivation function, retinoid receptors exert potent trans-repression function, which also plays an important role in mediating the diverse function of retinoids. Retinoid receptors, in response to their ligands, can inhibit the effect of TPA by repressing the transcriptional activity of AP-1 (20). The mechanism by which ligand-activated retinoid receptors repress AP-1 activity remains largely unknown, although a direct protein-protein interaction between retinoid receptors and AP-1 (20) and a competition for a common coactivator (21) have been proposed. Nevertheless, the interaction between membrane and retinoid receptor signaling pathways may represent an important mechanism by which retinoids exert their potent anti-neoplastic effect.

To further understand the growth regulatory effect of TPA and its interaction with retinoid signaling, we evaluated the interaction of TPA and trans-RA on growth of several human lung cancer and breast cancer cell lines and the underlying molecular mechanisms. Our results demonstrated that TPA exhibited different effects on growth of these cancer cell lines. TPA induced growth arrest of lung cancer cell lines H460 and H292 through either induction of p21WAF1 expression and ERK activity and/or inhibition of Cdk2 expression. In contrast, TPA enhanced proliferation of ZR75-1 breast cancer cells through induction of JNK and AP-1 activity. When the effect of
trans-RA on TPA activity was studied, we observed that it could additively increase the growth inhibitory effect of TPA in lung cancer cells, mainly due to its repression of constitutive AP-1 activity in the cells rather than its modulation of TPA-induced P21\(^{\text{WAF1}}\) expression and ERK activity. In contrast, trans-RA abolished the growth-stimulatory effect of TPA by repressing TPA-induced AP-1 activity in a JNK-independent mechanism in breast cancer cells. These results demonstrate that two potent growth regulators, TPA and trans-RA, play a critical role in regulating cancer cell growth and that trans-RA modulates TPA activity through its interaction with TPA-induced JNK/AP1 pathway but not TPA-induced ERK/p21\(^{\text{WAF1}}\) pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The non-small cell lung cancer cell lines H460 and H292 and breast cancer cell lines ZR75-1 and T47D were obtained from American Type Culture Collection (ATCC). They were grown in RPMI 1460 medium supplemented with 10% fetal calf serum (FCS).

**Growth Inhibition Assay**—Cells were seeded at a density of 1,000 cells per well in 96-well plates. One day later, the desired volume of TPA was added to the cells to achieve a final concentration of 0.001–10 nM. 1×10\(^5\) trans-RA was analyzed alone or in combination with various TPA concentrations. The control cells received vehicle (ethanol). Media and retinoids were changed every 48 h. Viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (22).

**Flow Cytometry Analysis**—Cells were trypsinized and collected by centrifugation at 2,000 rpm for 5 min. The cell pellets were then resuspended in 1 ml PBS and fixed in 70% ice-cold ethanol and kept in a freezer overnight. Fixed cells were centrifuged, washed once in PBS, and then resuspended in 100 μl of phosphate-citrate buffer (192 parts of 0.2 M Na\(_2\)HPO\(_4\) and 8 parts of 0.1 M citric acid, pH 7.8) for 30 min at room temperature to wash out any degraded DNA from apoptotic cells. The cells were then collected by centrifugation at 2,000 rpm, and the cell pellets were washed twice with PBS and resuspended in PBS containing 50 μg/ml propidium iodide (Sigma) and 100 μg/ml DNA-free RNase A (Roche Molecular Biochemicals). The cell suspension, protected against light, was incubated for 30 min at 37 °C and then analyzed using the FACS Citer-plus Flow cytometer.

**RNA Preparation andNorthern Blot Analysis**—For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride/ultracentrifugation method (22). About 30 μg total of RNAs from different cell lines were fractionated on 1% agarose gels, transferred to nitrocellulose membrane. Immunoblotting was carried out using anti-p21\(^{\text{WAF1}}\) (Santa Cruz), anti-p53 (Oncogene Inc.), and anti-Cdk2 (Santa Cruz) antibodies in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Detection was performed with an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). Anti-a-tubulin antibody (Sigma) was used as a control for protein loading.

**Protein Kinase Assays**—Cells were seeded in six-well plates 2 days prior to the analysis to provide ~80% confluent preparations. After treatment with different agents, cells were washed twice with ice-cold PBS solution and suspended in lysis buffer (25 mM HEPES, pH 7.7, 0.3 mM NaCl, 15 mM MgCl\(_2\), 0.1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotonin, 20 mM β-glycerophosphate, 0.1 mM Na\(_2\)VO\(_4\)). The Jun kinase assay was performed according to the method described previously (23). Briefly, 50 μg of whole cell lysate were mixed with 10 μg of glutathione S-transferase-c-Jun (1–223) (GST-c-Jun) and rotated for 3 h at 4 °C. GST-c-Jun proteins were purified from *Escherichia coli* and bound to agarose beads (Sigma). The beads were then washed twice and incubated with 20 μl of kinase reaction buffer (20 mM HEPES, pH 7.7, 20 mM MgCl\(_2\), 20 mM β-glycerophosphate, 20 mM p-nitrophosphinyl phosphate, 0.1 mM Na\(_2\)VO\(_4\), 2 mM dithiothreitol, 20 μM ATP, and 5 μCi of \(\gamma^{32}\)P[ATP] for 20 min at 30 °C. For ERK assay, 50 μg of whole cell extract was immunoprecipitated with anti-ERK2 antibody, which exhibit cross-reactivity with ERK1 (Santa Cruz) for 2 h at 4 °C. Immunoprecipitates were then washed and incubated with 20 μl of kinase reaction buffer containing 1 μg/reaction of myelin basic protein (MBP, Sigma) as substrate. Reactions were stopped by adding 15 μl of SDS-loading buffer containing 10% β-mercaptoethanol. Phosphorylated GST-c-Jun and MBP proteins were eluted by boiling the samples for 5 min, and resolved on 10% and 15% SDS-polyacrylamide gel electrophoresis, respectively.

**Transient Transfection Assays**—Cells were seeded in six-well culture plates at 5 × 10\(^4\) cells/well. A modified calcium phosphate precipitation procedure was used for transient transfection as described elsewhere (24). Briefly, 250 ng of reporter plasmid (73Col-CAT) (25, 26) and 250 ng of β-galactosidase expression vector (pβgal 110, Amersham Pharmacia Biotech) were mixed with carrier DNA (pBluescript) to 2.5 μg total of DNA/well. The day after transfection (18 h), cells were incubated in a medium containing 0.5% charcoal-treated FCS with trans-RA at the indicated concentrations and/or TPA (100 ng/ml) for an additional 24 h.

**RESULTS**

**Effect of TPA and trans-RA on the Growth of Lung and Breast Cancer Cells**—We investigated the growth inhibitory effect of TPA and trans-RA on a number of human cancer cell lines, including lung cancer cell lines H460 and H292 and breast cancer cell lines ZR75-1 and T47D. As shown in Fig. 1, TPA inhibited the growth of both lung cancer cell lines by 80% at 1 or 10 nM. TPA, however, did not exhibit any inhibitory effect on growth of ZR75-1 and T47D breast cancer cells over a broad range of concentrations from 0.001 to 10 nM. Interestingly, TPA at 10 nM, enhanced the growth of ZR75-1 cells but not T47D cells. These data demonstrate that the effect of TPA on the growth of cancer cells is cell type-dependent, consistent with previous observations (27–30). In contrast to TPA, trans-RA showed growth inhibition on all cell lines investigated. In lung cancer cells H460 and H292, the percentage of inhibition was about 40 and 20%, respectively, whereas in breast cancer cells ZR75-1 and T47D trans-RA showed 60 and 50% inhibition, respectively (Fig. 1). When trans-RA was used in combination with TPA, an additive growth inhibitory effect was observed in H460 and H292 cells when low concentrations of TPA (0.01 nM and 0.1 nM) were used. Interestingly, the
growth stimulatory effect of TPA on ZR75-1 cells was completely abolished by trans-RA (Fig. 1). Thus, trans-RA could enhance TPA-induced growth inhibition, but antagonizes TPA-induced cell proliferation.

**Regulation of Cell Cycle Progression by TPA and trans-RA**—To determine how TPA regulates growth of H460, H292, ZR75-1, and T47D cells, we investigated their cell cycle progression in response to TPA. The DNA content analysis showed that H460 and H292 cells underwent a stable G0/G1 arrest following 15 h of TPA treatment (Fig. 2, A and B). The entry of these cells into S phase was suppressed, while G0/G1 population was increased from 52 to 75%, and from 51% to 72%, respectively (Fig. 2, A and B, Table I). This data suggests that the growth inhibitory effect of TPA on these lung cancer cells is mainly due to its effect on cell cycle progression. When ZR75-1 and T47D breast cancer cells were analyzed, we observed a decrease in G0/G1 cell population (Fig. 2, A and B). This decrease was more apparent in ZR75-1 cells, with a percentage of cells in the G0/G1 phase decreasing from 75 to 43% when they were treated with 10 nM TPA for 15 h (Fig. 2, A and B, Table I). At the same time an increase S phase cell population of 37 and 16% was observed in ZR75-1 and T47D cells, respectively. When the effect of trans-RA on cell cycle progression was analyzed, trans-RA alone did not show significant changes of G0/G1 arrest of H460, H292, and ZR75-1 cells, except a slight increase (5%) observed in T47D cells (Table I). When trans-RA was used together with TPA, it slightly increased TPA-induced G0/G1 in H460 and H292 cells (Table I). However, the inhibitory effect of TPA on G0/G1 phase in ZR75-1 and T47D cells was largely blocked by trans-RA (Table I). In ZR75-1 cells, in the absence of trans-RA, TPA decreased G0/G1 phase from 75 to 43%, which was reverted to 55% when trans-RA was present. The effect of TPA on G0/G1 phase in T47D cells was completely abolished by trans-RA. These data demonstrate that trans-RA could inhibit the effect of TPA on cell cycle progression of ZR75-1 and T47D cells.

**Effect of TPA on Gene Expression**—To obtain insight into the molecular mechanism by which TPA regulates cell cycle progression of cancer cells, we examined the effect of TPA on p21<sup>WAF1</sup>, p53 and Cdk2 gene expression. When expression of p21<sup>WAF1</sup>, an inhibitor of cyclin-dependent kinases (31, 32), was determined by Northern blot analysis, we observed that it was rapidly and strongly induced by TPA in H460, H292, ZR75-1, and T47D cells (Fig. 3, A and B). Interestingly, when expression of p21<sup>WAF1</sup> was analyzed by Western blotting, we found that it was only strongly induced in H292 and H460 cells (Fig. 4A). Slight induction of p21<sup>WAF1</sup> was observed in T47D cells, whereas ZR75-1 cells did not show any expression of p21<sup>WAF1</sup> either in the absence or in the presence of TPA (Fig. 4B). This observation suggests that p21<sup>WAF1</sup> expression is also regulated by a post-transcriptional regulatory mechanism. A 12-h treatment increased p21<sup>WAF1</sup> expression by about 8-fold in H460 cells and 12-fold in H292 cells (Fig. 4A). There was no evidence for p53 induction by TPA in these cell lines (Fig. 4A). The fact that p21<sup>WAF1</sup> was induced in H460 and H292 cells, in which p53 was not expressed or induced indicates that TPA-induced p21<sup>WAF1</sup> is p53-independent. We also examined the effect of TPA on expression of Cdk2 gene, which is also known to play a critical role in G0/G1 progression (13). The Cdk2 gene was highly expressed in all cell lines investigated (Fig. 4, A and B). However, TPA treatment for 12 h strongly inhibited expression of Cdk2 in H460 cells (Fig. 4A), while it had no effect in H292, ZR75-1, and T47D cells (Fig. 4, A and B). These data suggest that induction of G0/G1 arrest by TPA in H292 cells is likely due to its effect on p21<sup>WAF1</sup>, whereas induction of p21<sup>WAF1</sup> and/or inhibition of Cdk2 expression may be responsible for TPA-induced G0/G1 arrest in H460 cells.

**Effect of TPA on c-Jun and c-Fos Gene Expression**—AP-1 is known to be associated with cell proliferation and it can be induced by TPA (1, 8). We then determined whether induction of AP-1 could account for enhancement of cell proliferation by TPA in ZR75-1 cells. As shown in Fig. 3B, expression of both c-Jun and c-Fos was strongly induced in ZR75-1 cells. Induction of c-Jun and c-Fos occurred as early as 30 min after TPA treatment. c-Jun was expressed in both H460 and H292 cells. However, its level of expression was not affected by TPA treatment. Expression of c-Fos in H460 and H292 cells was not influenced by TPA either (Fig. 3A). These data, therefore, suggest that induction of c-Jun and c-Fos may contribute to TPA-induced cell proliferation in ZR75-1 cells.

**Induction of ERK by TPA Is Mainly Responsible for p21<sup>WAF1</sup> Induction**—To study how TPA regulates p21<sup>WAF1</sup> expression and whether trans-RA modulates TPA activities in lung and breast cancer cell lines, we evaluated ERK activity that is known to regulate expression of p21<sup>WAF1</sup> (12). We examined the phosphorylation of MBP after immunoprecipitation of the whole cell extracts with anti-ERK2 antibody to determine ERK activity. As shown in Fig. 5, treatment of H460 cells with TPA for 30 min strongly induced ERK activity, while treatment with trans-RA did not show a clear effect on this activity. When trans-RA and TPA were used together TPA-induced ERK activity was not affected (Fig. 5). In ZR75-1 cells, ERK activity

![Figure 2](image-url)
was slightly induced by TPA. Again, trans-RA did not show any effect on TPA-induced ERK activity in these cells. To determine whether induction of ERK activity by TPA is responsible for p21WAF1 induction, we examined the effect of PD98059, a specific inhibitor of Raf/ERK pathway, on p21WAF1 expression in H460 and ZR75-1 cells (Fig. 6). PD98059 alone (50 μM) did not show any effect on the expression of p21WAF1. However, when PD98059 was used together with 10 nM TPA, induction of p21WAF1 by TPA was completely inhibited in both cell lines (Fig. 6). This suggests that induction of ERK is mainly responsible for p21WAF1 induction. Treatment of H460 cells with trans-RA for 24 h did not show a clear effect on p21WAF1 expression, consistent with the observation that trans-RA could not affect TPA-induced ERK activity. Thus, trans-RA has no effect on TPA-induced ERK/p21WAF1 pathway.

Induction of c-Jun and JNK by TPA Modulates Its Effect on the Growth of Cancer Cells—The above data demonstrate that TPA was able to induce c-Jun and c-Fos expression in ZR75-1 cells (Fig. 3B), suggesting that signaling that leads to AP-1 induction is functional in these cells. Transcriptional regulation of c-Jun expression is mainly mediated by a TPA-response element in its promoter, which binds to c-Jun/ATF-2 heterodimer (33). ATF-2 and c-Jun are activated mainly by JNK. The fact that c-Jun expression was rapidly induced by TPA in ZR75-1 cells (Fig. 3B) suggests that TPA may induce JNK in this cell line. To investigate this possibility, we analyzed JNK activation in H460 and ZR75-1 cells. JNK activity was determined by examination of the phosphorylation of GST-c-Jun in whole cell extracts prepared from H460 and ZR75-1 cells treated with different agents. As shown in Fig. 7, treatment of ZR75-1 cells with 10 nM TPA for 30 min strongly induced JNK activity in these cells. However, the same treatment failed to activate JNK in H460 cells. As a control, UV stimulation exhibited a strong activation of JNK in both cell lines. These data suggest that induction of c-Jun by TPA in ZR75-1 cells is likely due to activation of JNK. trans-RA has been shown to inhibit

## Table I

### Effect of TPA and RA on cell cycle distribution of human cancer cell lines

| Cell cycle phase | H460 | H292 | ZR75-1 | T47D |
|------------------|------|------|--------|------|
|                  | Control | TPA  | RA    | TPA + RA | Control | TPA  | RA    | TPA + RA | Control | TPA  | RA    | TPA + RA |
|                  | %     | %    | %     | %      | %     | %    | %     | %       | %     | %    | %     | %       |
| G0/G1            | 52    | 79   | 53    | 84     | 51    | 72   | 54    | 81      | 75    | 43   | 72    | 55      |
| S                | 36    | 6    | 32    | 15     | 36    | 7    | 27    | 8       | 38    | 55   | 20    | 45      |
| G2/M             | 12    | 5    | 15    | 7      | 13    | 21   | 19    | 11      | 7     | 2    | 8     | 0       |

*Cell cycle distribution of cells treated with 10^{-6} M trans-RA and/or 10 nM TPA. Values represent the result of flow cytometry experiments. Representative histograms for cells treated with 10 nM TPA are shown in Fig. 2A.*

![Fig. 2. Regulation of c-Jun, c-Fos, and p21WAF1 expression by TPA. A, lung cancer cell lines. B, breast cancer cell lines. Cells were treated with 10 nM TPA at the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated genes by Northern blotting. Expression of β-actin is shown to ensure that equal amounts of RNAs were used, not treated with TPA.](image)

![Fig. 3. Regulatio of c-Jun, c-Fos, and p21WAF1 expression by TPA. A, lung cancer cell lines. B, breast cancer cell lines. Cells were treated with 10 nM TPA at the indicated times, and total RNAs were prepared and analyzed for the expression of the indicated genes by Northern blotting. Expression of β-actin is shown to ensure that equal amounts of RNAs were used, not treated with TPA.](image)

![Fig. 4. Analysis of TPA effect on p21WAF1, p53, and Cdk2 expression by Western blot. A, lung cancer cells. B, breast cancer cells. Cell extracts were prepared from the indicated cell lines treated with 10 nM TPA at the indicated times, and total RNAs were analyzed for the expression of p21WAF1, p53, and Cdk2. Expression of α-tubulin gene is shown as a control for protein loading. –, not treated with TPA.](image)

![Fig. 5. Effect of TPA and trans-RA on ERK activity. ERK activity was determined in H460 lung and ZR75-1 breast cancer cells. Cells maintained in 0.5% FCS were treated for 30 min with TPA (10 nM) or UV (100 J/m²). trans-RA was used at 10^{-6} M. When trans-RA was used in combination with TPA, cells were first incubated for 24 h with trans-RA before TPA addition. Whole cell extracts were prepared as described under “Experimental Procedures.” Kinase activity was measured via the phosphorylation of the MBP protein. Control, untreated cells.](image)
Regulation of Phorbol Ester Activities by Retinoic Acid

In this study, we investigated the effect of two potent growth regulators, TPA and trans-RA, on the growth of lung (H460, H292) and breast (ZR75-1, T47D) cancer cell lines and their interaction. Our results demonstrate that TPA exerts either inhibition or stimulation of cancer cell proliferation, whereas trans-RA shows various degrees of growth inhibition in all cell lines investigated (Fig. 1). When trans-RA was used together with TPA an additive growth inhibitory effect was observed in H460 and H292 cells, whereas it completely abolished the growth-stimulatory effect of TPA on ZR75-1 breast cancer cells. We also show that induction of G1/G0 arrest by TPA in H460 and H292 cells is accompanied by induction of p21WAF1 due to activation of ERK pathway (Fig. 5) and/or inhibition of Cdk2 gene expression, whereas stimulation of cell proliferation in ZR75-1 cells by TPA is associated with induction of c-Jun and c-Fos expression and JNK activation (Fig. 3B, Fig. 7). Although trans-RA did not interfere with TPA-induced p21WAF1 expression and ERK activity, it strongly inhibited TPA-induced AP-1 activity without affecting TPA-induced JNK activity (Figs. 6, 7, and 8).

The growth inhibitory effect of TPA on H460 and H292 cells is mainly due to arrest of these cells in G1/G0 phase (Fig. 2, A and B, Table I), due to induction of p21WAF1 expression in H292 cells and/or inhibition of Cdk2 expression in H460 cells (Figs. 3 and 4). P21WAF1 can interact with cyclin-Cdk complexes and is capable of inhibiting kinase activities associated with these complexes (31, 32). A major target of p21WAF1 inhibition is the cyclin-Cdk2 kinase complex whose activity is required for G1/S progression into S phase (13, 14). TPA has been shown to induce p21WAF1 in a variety of cell types (35, 36). Inhibition of Cdk2 expression by TPA has also been observed during the differentiation of HL60 leukemia cells (37). Thus, the increase of p21WAF1 in H292 cells and/or the decrease of Cdk2 expression in H460 cells upon TPA treatment may be sufficient to inhibit kinase activity required for G1/S progression into S phase. These results, taken together with previous findings (35–37), suggest that induction of p21WAF1 and/or inhibition of Cdk2 expression may play a causative role in TPA-induced growth arrest. Interestingly, p21WAF1 messenger was also highly induced by TPA in ZR75-1 cells (Fig. 3B). However, we did not detect any p21WAF1 protein product (Fig. 4B). A previous study (38) demonstrated that expression of p21WAF1 often involves a post-transcriptional mechanism. The inability of ZR75-1 cells to express p21WAF1 protein product suggests that p21WAF1 transcript may be unstable in these cells. Although p21WAF1 was also induced in T47D cells (Figs. 3 and 4), the degree of induction is much less than those observed in H460 and H292 cells, and may not be enough to confer a G1 arrest by TPA in the cells (Fig. 2 and Table I). Induction of p21WAF1 by DNA damage requires p53 (39). However, under many experimental conditions p21WAF1 can be induced through p53-independent pathways (35–37). Our observation that p21WAF1 was induced by TPA in H460 and H292 cells without affecting p53 expression (Figs. 3A and 4A) suggests that TPA induced p21WAF1 expression is p53-independent. In studying possible signaling pathway leading to p21WAF1 induction in H460 cells, we found that TPA strongly activated ERK kinase activity, while it had no effect on JNK activity (Figs. 5 and 7). Our observation that PD98059, a specific inhibitor of Raf/ERK pathway, abolished p21WAF1 induction by TPA (Fig. 6) suggests that ERK pathway is responsible for TPA-induced p21WAF1 in both H460 and ZR75-1 cells. A similar study also showed that induction of p21WAF1 by TPA in SKBR3 breast cancer and LNCaP prostate cancer cells is attributed to stimulation of Raf-1/MEK pathway (40).
Our results also demonstrate that TPA can stimulate proliferation of ZR75-1 breast cancer cells (Fig. 1). Although TPA acts as a potent inducer of growth arrest of many cancer cell types, the mitogenic effect of TPA has been also described (5, 7). TPA inhibited the growth of malignant melanoma cells, while the growth of normal melanocytes was stimulated (41). Similarly, two NIH3T3 clones, N3T3 and P-3T3, showed opposite response to TPA (42); TPA inhibited the growth of N-3T3 cells, while it stimulated the growth of P-3T3 cells. Our present study further demonstrates the diverse functions of TPA, which is likely determined by the cell context, which in turn dictates the biological outcome of TPA. The molecular mechanism by which TPA induces growth arrest is well studied. However, how TPA stimulates cell proliferation is less understood. When we investigated the effect of TPA in ZR75-1 cells, we found that it strongly induced expression of c-Jun and c-Fos (Fig. 3A). Such an effect was not seen in H460 cells (Fig. 3A). C-Jun and c-Fos, the components of AP-1, act as transcriptional factors for numerous genes, and overexpression of these genes is often associated with cell proliferation and malignant transformation (8). Thus, induction of AP-1 activity by TPA may contribute to its growth stimulatory effect in ZR75-1 cells. Our results also demonstrate that induction of c-Jun and c-Fos expression by TPA is likely due to activation of JNK, which is known to activate c-Jun promoter through phosphorylation of c-Jun and ATF2 that bind to a TPA-response element in the c-Jun promoter as heterodimer (33). Interestingly, activation of JNK was not observed in H460 cells (Fig. 7), which could explain the inability of TPA to induce c-Jun expression in these cells. Taken together, our results demonstrate that the pleiotropic effects of TPA are mediated by multiple signaling pathways whose operation is largely determined by the cellular context.

Previous studies have demonstrated that trans-RA could effectively counteract TPA effects (20, 25, 26, 43). trans-RA could prevent transformation of JB6 mouse epidermal cells promoted by TPA (43) and counteract the effect of TPA on expression of fibronectin gene in fibroblasts (44), transglutaminase 1 gene in keratinocytes (45), as well as collagenase (46), stromelysin (47), and ornithine decarboxylase (48). In this study, we found that trans-RA exerted different effects on TPA activities in different cell lines. In H460 and H292 cells, pretreatment of the cells with trans-RA increased the growth inhibitory effect of TPA. An additive effect was observed when 1 nM or less TPA was used. In contrast, trans-RA antagonized the growth stimulatory effect of TPA on ZR75-1 cells. In the absence of trans-RA, TPA enhanced proliferation of ZR75-1 cells, which was almost completely abolished when trans-RA was added (Fig. 1). Although p21WAF1 promoter contains a RA response element (49) we did not observe any effect of trans-RA on p21WAF1 expression (Fig. 6). This suggests that the growth inhibitory effect of trans-RA is unlikely due to induction of p21WAF1. In studying the antagonism effect of trans-RA on TPA activity in ZR75-1 cells, we found that trans-RA could effectively inhibit TPA-induced AP-1 activity (Fig. 8A). TPA could induce endogenous AP-1 activity as demonstrated by our observation that TPA strongly induced collagenase promoter activity in ZR75-1 cells (Fig. 8A). The TPA-induced AP-1 activity in ZR75-1 cells was largely inhibited by trans-RA. This is consistent with previous observations showing that trans-RA could antagonize AP-1 activity in HeLa cells (25) and suggests that trans-RA may antagonize the growth-stimulatory effect of TPA in ZR75-1 cells through repression of TPA-induced AP-1 activity. Interestingly, trans-RA could also inhibit the constitutive AP-1 activity in H292 cells (Fig. 8B), suggesting that the growth inhibitory effect of trans-RA in these cells may be in part due to inhibition of AP-1 activity which may contribute to the additive growth inhibitory effect of TPA and trans-RA combinatory treatment observed in these cells (Fig. 1). A previous study demonstrated that trans-RA inhibited JNK activity in HeLa cells (34). However, we did not detect any effect of trans-RA on TPA-induced JNK activity in ZR75-1 cells (Fig. 7). Thus a mechanism other than inhibition of JNK activity may be responsible for inhibition of AP-1 activity by trans-RA.

Together, our results demonstrate that TPA could exert mitogenic or anti-mitogenic effect through different signaling transduction pathways in a cell type specific manner. trans-RA may enhance anti-mitogenic effect of TPA and antagonize its mitogenic effect through inhibition of AP-1 activity. These two potent regulators of cell growth, through their interaction, are expected to play a critical role in the regulation of cancer cell growth.

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