Title

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Journal

Cancer Research, 57(16)

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Publication Date

1997-08-15

Peer reviewed

Retinyl Methyl Ether Down-Regulates Activator Protein 1 Transcriptional Activation in Breast Cancer Cells[1]

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ABSTRACT

Retinyl methyl ether (RME) is known to prevent the development of mammary cancer. However, the mechanism by which RME exerts its anticancer effect is presently unclear. The diverse biological functions of retinoids, the vitamin A derivatives, are mainly mediated by their nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are ligand-dependent transcriptional factors that either activate gene transcription through their binding to retinoic acid response elements or repress transactivation of genes containing the activator protein 1 (AP-1) binding site. Previous studies demonstrated that RME can modulate transcriptional activity of retinoid receptors on retinoic acid response elements, suggesting that regulation of retinoid receptor activity may mediate the anticancer effect of RME. In this study, we present evidence that RME can down-regulate AP-1 activity induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, insulin, growth factors, and the nuclear proto-oncogenes c-Jun and c-Fos. Transient transfection assays demonstrate that inhibition of AP-1 activity occurs on the human collagenase promoter containing an AP-1 binding site or the thymidine kinase promoter linked with an AP-1 binding site. In HeLa cells, the inhibition is observed when RAR-α and/or RXR-α but not RAR-β or RAR-γ expression vectors are cotransfected, whereas the endogenous retinoid receptors in breast cancer cells T-47D and ZR-75-1 were sufficient to confer the inhibition by RME. Furthermore, using gel retardation assay, we show that 12-O-tetradecanoylphorbol-13-acetate- and epidermal growth factor-induced AP-1 binding activity in breast cancer cells is inhibited by RME. These results suggest that one of the mechanisms by which RME prevents cancer development may be due to the repression of AP-1-responsive genes.

INTRODUCTION

Retinoids are a group of natural and synthetic vitamin A analogues that exert profound effects on many biological processes, including cell proliferation and differentiation, vision, reproduction, morphogenesis, and pattern formation (1–2). They are promising agents for the prevention of human cancers (3, 4), including cancers of the breast, skin, head and neck, lung, prostate, and bladder. Retinyl ethers represent an unique class of vitamin A analogues with effective cancer-preventive function (5–7). RME[3] is significantly more active than RA in inhibiting hyperplasia induced by N-methyl-N'-nitro-N-nitrosoguanidine in mouse prostate organ culture (8) and in inhibiting hyperplasia and squamous metaplasia induced by different carcinogens in organ cultures of hamster trachea (9). RME and related compounds prevent the development of mammary tumors induced in

rats by different carcinogens (5–7). Studies of their pharmacological distribution in tissue reveal a correlation between their accumulation and their cancer-preventive effect (6, 10). However, the molecular mechanism by which RME exerts its anticancer effect remains unknown.

The effects of retinoids are thought to result from modulation of gene activity by at least two distinct classes of nuclear receptors: (a) RARs, which bind and are activated by trans-RA or 9-cis-RA; and (b) RXRs, which bind only to the 9-cis-RA isomer (11–13). These receptors exist as major subtypes α, β, and γ, from which multiple isoforms can be generated as a result of alternative splicing and differential promoter usage (11–13). RARs and RXRs, like other members of the steroid/thyroid hormone superfamily, have similar domain structures: an autonomous activation domain (AF-1) in the amino-terminal part of the receptor (A/B region), and a hormone-binding domain (E region) responsible for ligand binding and receptor transactivation (11, 13). RARs and RXRs function as ligand-activated transcription factors that bind to specific RAREs on target genes as homodimeric or heterodimeric complexes and positively or negatively regulate the expression of the genes (11–14). Retinoid receptor-mediated transcriptional activation can contribute to the anticancer activity of retinoids. A well-known example is the effect of RA-activated RAR-α in the treatment of acute promyelocytic leukemia, whereby the proliferating nondifferentiated leukemic promyelocyte matures into a nonproliferating differentiated granulocyte on retinoid treatment (15). Furthermore, ligand-activated RAR-β may be required for anticancer activity of vitamin A analogues in breast cancer cells (16) or in patients with oral lesions (17).

In addition to the regulation of RARE-containing genes, retinoid receptors in response to RA can inhibit the effect of the tumor promoter TPA by repressing the transcriptional activity of AP-1 (14, 18–20). AP-1 constituents are products of the cellular proto-oncogenes c-Jun and c-Fos that bind as either c-Jun homodimers or c-Jun/c-Fos heterodimers to TREs, resulting in a stimulation of TRE-dependent transcription (21). Unlike the effect of retinoid receptors on RAREs, inhibition of AP-1 activity by retinoid receptors does not involve a direct retinoid receptor-RARE interaction, but rather a protein-protein interaction that may result in the inhibition of AP-1 DNA binding. Recent results suggest that the molecular basis of receptor-mediated inhibition of AP-1 transcriptional activation may be due to competition for a common coactivator such as CREB (22). CBP is required for transcriptional activation by both CREB and AP-1 (23–25) as well as by nuclear receptors such as RARs (22). Such interaction between membrane and nuclear receptor signaling pathways mediated by RAR/AP-1 interaction through CBP may be important in understanding the mechanisms underlying the potent antineoplastic effects of retinoids. Because many of the AP-1-responsive genes are involved in cancer cell proliferation and transformation (21), the anti-AP-1 activity of retinoids could contribute to their chemopreventive effect by inhibiting the processes of cell proliferation and cell transformation. This is supported by a recent observation that retinoids that selectively inhibit AP-1 activity and cannot induce transactivation of RARE-containing genes were able to inhibit TPA-induced transformation and the clonal growth of the promotion-

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sensitive mouse epidermal JB6 cell line (26). In addition, anti-AP-1 retinoids inhibit the proliferation of lung and breast cancer cells (27, 28).

We previously showed that RME could influence the transcriptional potential of RARs and RXRs (29), suggesting that RME may exert its anticancer activity through regulation of retinoid receptor activity. To further extend our understanding of the anticancer effect of RME, we investigated the possibility that RME regulates the anti-AP-1 function of retinoid receptors. Our data demonstrate that RME is an effective inhibitor of AP-1 activity induced by tumor promoter TPA, growth factors, and the nuclear proto-oncogenes c-Jun and c-Fos and suggest that one of the mechanisms by which RME exerts its cancer-preventive action may be due to the repression of AP-1-responsive genes.

MATERIALS AND METHODS

Cell Culture and Reagents. HeLa cells were grown in DMEM supplemented with 10% FCS. Breast cancer cell lines T-47D and ZR-75-1 were maintained in RPMI 1640 supplemented with 10% FCS. Trans-RA, TPA, insulin, and EGF were purchased from Sigma Chemical Co. (St. Louis, MO). Synthesis of RME was accomplished as described previously (7). 9-cis-RA was kindly provided by Dr. M. I. Dawson (SRI International, Menlo Park, CA).

Plasmids. The collagenase promoter reporter construct (−73Col-CAT) was described previously (18, 30). The TRE_2-tk-CAT reporter was constructed by inserting two copies of AP-1 binding site (TRE; 5'-AGCTTGGTGACT-CATCCGGATCCGGATGAGTGAGTCACCAAGCT-3') present in the collagenase promoter region into the BamHI site of pBL-CAT$_2$ (31). Expression vectors for RAR-α, RAR-β, RAR-γ, RXR-α, c-Jun, and c-Fos were described previously (18, 32).

Transient Transfection and CAT Assay. HeLa cells were plated at a density of 10^5 cells/well in 24-well plates and transfected by a modified calcium phosphate precipitation procedure (18). The precipitates contained 100 ng of reporter plasmid, 100 ng of β-gal expression vector (pCH 110; Pharmacia), and variable amounts of receptor expression vectors. The total amount of transfected DNA was adjusted with pBluescript (Stratagene) to 1 μg. For cancer cell lines, cells were seeded at a density of 5×10^5 cells/well in 6-well plates. The precipitates contained 250 ng of reporter plasmid and 400 ng of β-gal expression vector. The total amount of transfected DNA was adjusted with pBluescript to 2.5 μg. Twenty-four h after transfection, cells were incubated in a medium containing 0.5% charcoal-treated FCS with retinoids and/or TPA (100 ng/ml), EGF (10 ng/ml), or insulin (10 μg/ml) for an additional 16 h. CAT activity was determined by using [^{3}H]acetyl-CoA as substrate. β-gal activities were measured to normalize transfection efficiency.

Preparation of Nuclear Extracts and Gel Retardation Assays. Nuclear extracts were prepared essentially according to the method described previously (33). Briefly, cells growing in 0.5% charcoal-treated FCS were treated with retinoids (10^{-6} M trans-RA or RME) and/or TPA (100 ng/ml) or EGF (10 ng/ml) for 6 h. Cells were washed with ice-cold PBS solution and scraped into PBS using a rubber policeman. The cells were pelleted by low-speed centrifugation and resuspended in 1 ml of buffer A [10 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$, and 2 mM $MgCl_2$]. After pelleting, the cells were lysed in 1 ml of buffer A containing 1% NP40 by 10–15 strokes using an ice-cold Dounce homogenizer. Nuclei were collected by centrifugation at 2000 × g and washed with 1 ml of buffer B [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT]. Nuclear proteins were extracted with 200 μl of high-salt buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT]. All buffers contained protease inhibitors, i.e., 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. To study AP-1 binding, 3 μg of nuclear extracts were analyzed by gel retardation assay using the ^{32}P-labeled AP-1 binding site as a probe as described previously (18). The AP-1 used in the experiments is the TRE present in collagenase promoter (TGACTCA). For control, binding of nuclear proteins to SP-1 (CCCGAGGTGGGCGGGTGAGGCC) was used. Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

RESULTS

Down-Regulation of TPA-induced Collagenase Promoter Activity by RME. Retinoid receptors, in response to their ligands, can down-regulate the activities of the tumor promoter TPA and oncogenes c-Jun and c-Fos (18–20). We have recently observed that RME can modulate transcriptional activity of retinoid receptors on RAREs (29). To determine whether RME could also influence the anti-AP-1 activity of retinoid receptors, the −73Col-CAT reporter gene (18, 30) was transfected with or without RARs (α, β, and γ) and/or RXR-α expression vectors into HeLa cells, which express very low levels of retinoid receptors (18). In these cells, the transactivation activity of the collagenase promoter can be induced by TPA due to the presence of an AP-1 binding site (TRE; Ref. 30). After transfection, cells were treated with 100 ng/ml TPA in the absence or presence of various concentrations (10^{-8}, 10^{-7}, and 10^{-6} M) of RME. For comparison,
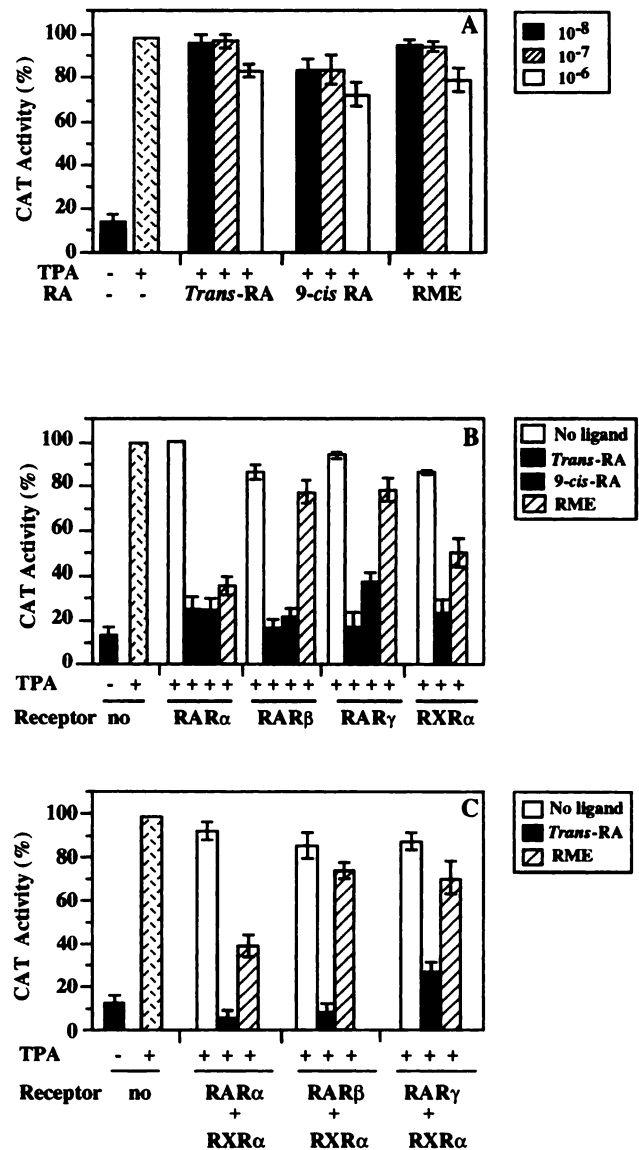

Fig. 1. Inhibition of TPA-induced collagenase promoter activity by RME. The −73Col-CAT reporter (100 ng) was cotransfected without (A) or with the indicated retinoid receptor expression vectors (200 ng; B and C) into HeLa cells. After transfection, cells were incubated in DMEM containing 0.5% FCS and treated with either trans-RA, 9-cis-RA, or RME and/or TPA (100 ng/ml). Twenty-four h later, the cells were harvested, and CAT activities were determined. β-gal activities were measured to normalize transfection efficiency. The mean of CAT activity in at least three independent experiments is shown (± SE).

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Fig. 2. Inhibition of TPA-induced TRE-tk-CAT activity by RME. The TRE_2-tk-CAT reporter (100 ng) was cotransfected with or without RAR-α or RXR-α expression vectors (200 ng) into HeLa cells. After transfection, the cells were incubated in DMEM containing 0.5% FCS and treated with either 10^{-6} M trans-RA, 9-cis-RA, or RME and/or TPA (100 ng/ml). CAT activities were measured as described in the legend to Fig. 1. Data shown represent mean of duplicate experiments (± SE).
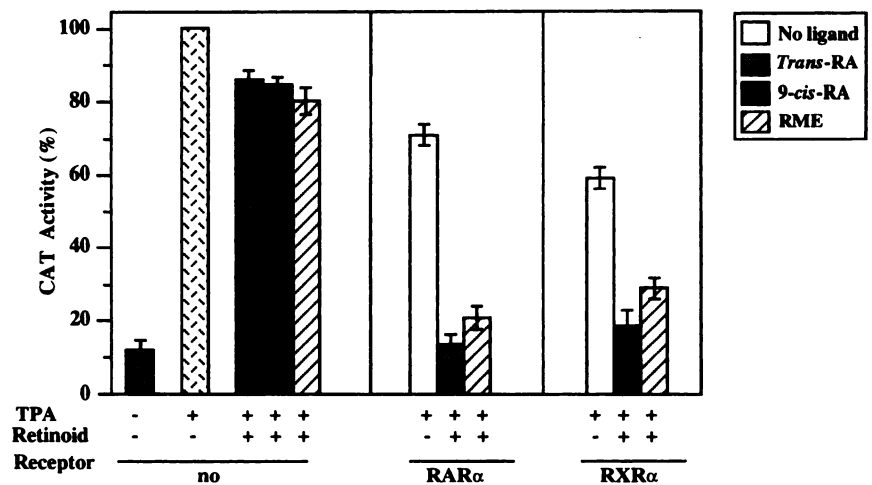
the effect of trans-RA and 9-cis-RA was examined. Treatment of HeLa cells with TPA led to a 7-fold induction of promoter activity (Fig. 1), presumably through induction of endogenous AP-1 activity (30). In the absence of cotransfected RA receptors, trans-RA, 9-cis-RA, or RME at 10^{-8} and 10^{-7} M did not show a clear effect on TPA-induced collagenase promoter activity (Fig. 1A). At 10^{-6} M, all three retinoids showed slight inhibition of TPA-induced reporter activity with 20, 30 and 25% inhibition, respectively (Fig. 1A). When RAR-α expression vector was cotransfected, both trans-RA and 9-cis-RA at 10^{-6} M completely inhibited TPA-induced collagenase promoter activity (Fig. 1B), as reported previously (18). Remarkably, RME at 10^{-6} M exhibited a 65% inhibitory effect (Fig. 1B). The inhibition by RME seems to be specific for RAR-α, because cotransfection of RAR-β or RAR-γ did not lead to inhibition (Fig. 1B). In comparison, trans-RA or 9-cis-RA exhibited strong inhibition under the same conditions, with 90 and 85% for RAR-β and 90 and 60% for RAR-γ, respectively. When RXR-α expression vector was cotransfected (Fig. 1B), both 9-cis-RA and RME showed inhibition (75 and 50%, respectively). RAR and RXR heterodimerization is required for efficient RARE binding and transactivation (11–13, 32). To investigate the possible effect of heterodimerization, each RAR expression vector was cotransfected with RXR-α (Fig. 1C). Our results demonstrate that cotransfection of RXR-α does not significantly influence

the inhibition profile of RME (Fig. 1C). Together, these data demonstrate that RME in the presence of RAR-α and/or RXR-α is an effective inhibitor of TPA-induced collagenase promoter activity.

To determine whether inhibition of TPA-induced activity by RME is mediated by the TRE site and not by other regions of the collagenase promoter, the TRE was cloned into pBL-CAT_2, which contains the tk promoter linked with the CAT gene (31). The resulting TRE_2-tk-CAT reporter was transiently transfected into HeLa cells. Treatment of HeLa cells with TPA (Fig. 2) significantly induced the reporter activity (an 8-fold induction) as that observed on −73Col-CAT (Fig. 1), suggesting that the reporter is responsive to the TPA-induced endogenous AP-1 activity. TPA-induced reporter activity was not clearly affected by either 10^{-6} M trans-RA, 9-cis-RA, or RME when the cells were treated with these compounds. However, all ligands showed a strong inhibitory effect on TPA-induced reporter activity when RAR-α or RXR-α expression vectors were cotransfected (Fig. 2). Under the conditions used, 10^{-6} M trans-RA completely inhibited TPA-induced reporter activity, whereas about 80% of TPA activity was repressed by 10^{-6} M RME in the presence of RAR-α (Fig. 2) and 50% in the presence of RXR-α. Similar to that observed on the collagenase promoter (Fig. 1B), cotransfection of either RAR-β or RAR-γ did not influence the inhibitory effect of

Fig. 3. Inhibition of c-Jun and c-Fos activity by RME. The TRE_2-tk-CAT reporter (100 ng) was cotransfected with c-Jun alone (50 ng) or together with c-Fos (50 ng) expression vectors in the absence or presence of RAR-α (200 ng) into HeLa cells. After transfection, cells were treated with 10^{-6} M trans-RA or RME. CAT activities were determined as described in the legend to Fig. 1. Data shown are representative of three independent experiments (± SE).
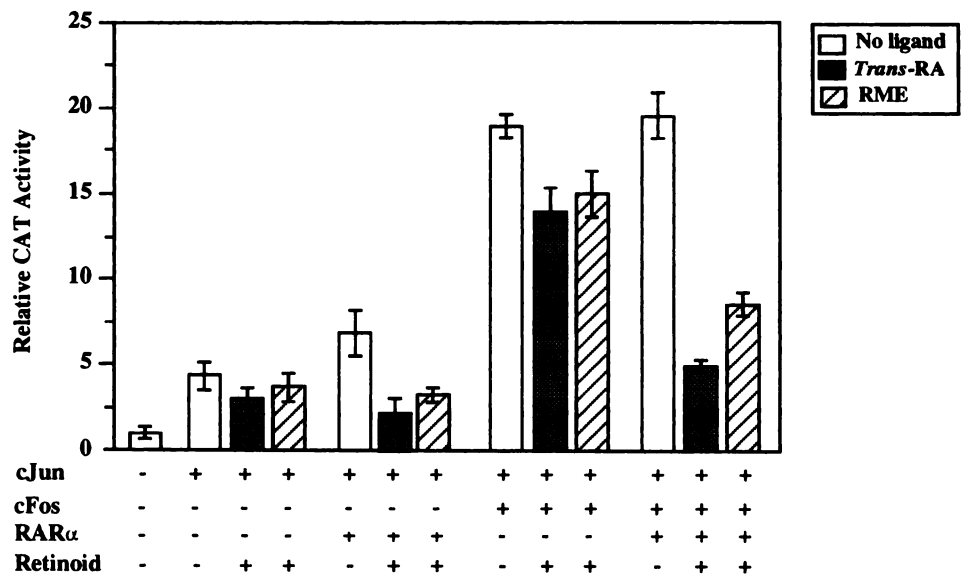
Fig. 4. Inhibition of TPA-induced collagenase promoter activity by RME in human breast cancer cells. A, T-47D cell line. B, ZR-75-1 cell line. The −73Col-CAT reporter (250 ng) was transfected into T-47D and ZR-75-1 cells. After transfection, cells were incubated in RPMI 1640 containing 0.5% FCS and treated with 10^{-6} M trans-RA or RME and/or TPA (100 ng/ml). CAT activities were measured as described in the legend to Fig. 1. Data shown are representative of three independent experiments (± SE).
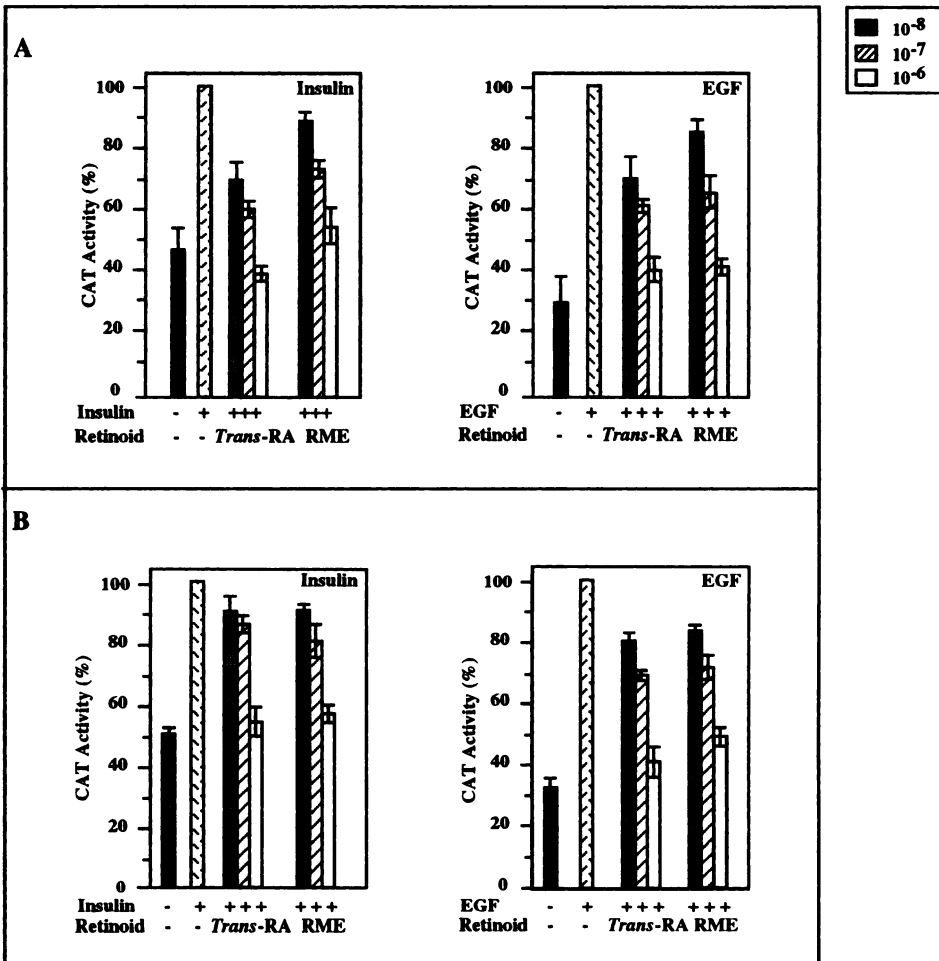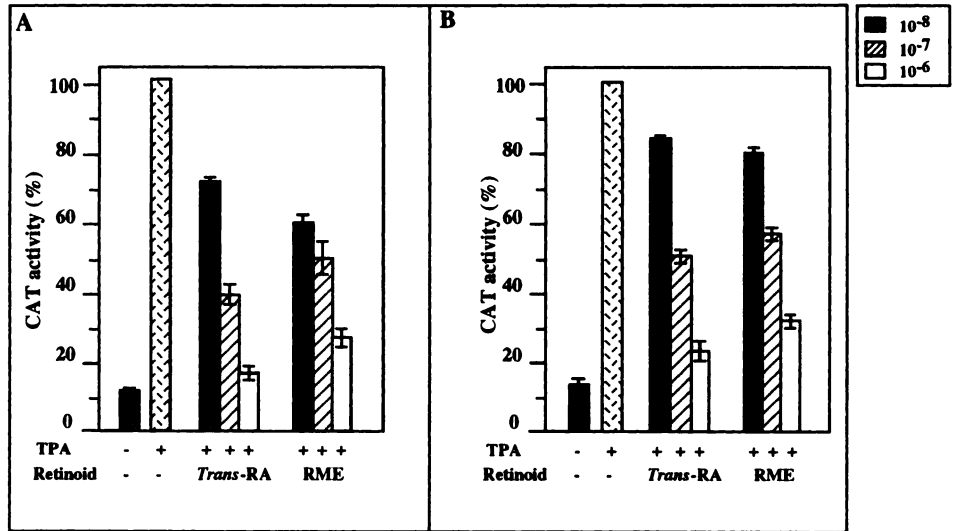
Fig. 5. Inhibition of peptide growth factors-induced collagenase promoter activity in human breast cancer cells. A, T-47D cell line. B, ZR-75-1 cell line. Transfection was carried out as described in the legend to Fig. 4. After transfection, cells were treated with either 10^{-6} M trans-RA or 10^{-6} M RME and/or insulin (10 μg/ml) or EGF (10 ng/ml). CAT activities were determined as described in the legend to Fig. 1. Data shown represent the mean of duplicate experiments (± SE).

RME (data not shown). Thus, inhibition by RME of TPA activity on the collagenase promoter is mediated by the TRE.

Effect of RME on the Nuclear Proto-Oncogenes c-Jun and c-Fos. The observation that TPA-induced TRE activity is inhibited by RME suggests that RME may inhibit the proto-oncogenes c-Jun and c-Fos that bind to the TRE (30). To evaluate this possibility, TRE$_2$-tk-CAT was cotransfected with either c-Jun alone or together with c-Fos into HeLa cells. An induction of reporter activity of about 4-fold

was observed when 100 ng of c-Jun expression vector was cotransfected (Fig. 3). When RAR-α expression vector was cotransfected, 50% of the c-Jun homodimer activity was repressed by 10^{-6} M RME. Cotransfection of c-Jun and c-Fos expression vectors largely increased TRE$_2$-tk-CAT reporter activity, with a 14-fold induction of reporter gene transcription (Fig. 3). The induced reporter activity was also repressed by RME in the presence of RAR-α. Together, these data demonstrate that RME in the presence of RAR-α can repress

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c-Jun homodimer- and c-Jun/c-Fos heterodimer-induced TRE activity. Thus, RME inhibits not only TPA-induced AP-1 activity but also the activity of the proto-oncogenes c-Jun and c-Fos.

Inhibition of AP-1 Activity by RME in Human Breast Cancer Cells. To determine whether RME could inhibit AP-1 activity in breast cancer cells, the −73Col-CAT was transiently transfected into T-47D and ZR-75-1 breast cancer cell lines, both of which express significant levels of RAR-α (16). Fig. 4 shows that treatment of the cells with TPA led to an increase of reporter gene transcription of about 7-fold in both T-47D (Fig. 4A) and ZR-75-1 cells (Fig. 4B). TPA-induced activity was inhibited by either *trans*-RA or RME in a concentration-dependent manner (Fig. 4, A and B). RME (10^{-7} M) inhibited the induced reporter activities in both cell lines by about 40%, whereas at 10^{-6} M, RME almost completely inhibited the TPA-induced reporter activity. Several peptide growth factors, such as EGF and insulin, exhibit strong mitogenic activity. They have been implicated in breast tumor growth and are thought to contribute to tumor malignancy (34, 35). To study whether RME could also inhibit peptide growth factor-induced AP-1 activity in breast cancer cells, the −73Col-CAT reporter was transfected into both T-47D and ZR-75-1 cells. Transfected cells were then treated with insulin (10 µg/ml) or EGF (10 ng/ml; Fig. 5). Treatment of T-47D cells (Fig. 5A) and ZR-75-1 cells (Fig. 5B) with either insulin or EGF resulted in about a 2–3-fold increase of the reporter activity. Similar to its effect on TPA-induced reporter activity, RME also strongly inhibited AP-1 activity induced by either insulin or EGF (Fig. 5, A and B). Thus, endogenous RARs in breast cancer cells are sufficient to mediate the inhibitory effect of RME on the AP-1 activity induced by a variety of stimuli.

Effect of RME on TPA- and EGF-induced AP-1 DNA Binding Activity. Inhibition of AP-1 binding by RARs has been suggested to account for the anti-AP-1 effect of retinoids (18–20). To investigate whether inhibition of AP-1 activity by RME was due to a decrease of AP-1 binding, nuclear proteins were prepared from HeLa cells treated with TPA (100 ng/ml) and/or 10^{-6} M RME. AP-1 binding was then determined by gel retardation assay using the radiolabeled TRE as a probe. Treatment of HeLa cells with TPA strongly enhanced AP-1 binding activity, whereas treatment with RME did not affect AP-1 binding (Fig. 6). When the cells were treated with both TPA and RME, we did not observe a clear inhibition of TPA-induced AP-1 binding activity. Because the inhibitory effect of RME requires retinoid receptors, we therefore examined whether RME could alter AP-1 binding properties in ZR-75-1 breast cancer cells, which express high levels of RAR-α (16). Similar to that observed in HeLa cells, treatment of ZR-75-1 cells with either TPA or EGF strongly induced AP-1 binding activity (Fig. 7). However, when ZR-75-1 cells were pretreated with 10^{-6} M RME, both TPA- and EGF-induced AP-1 binding activities were repressed. These data suggest that the inhibition of expression of AP-1 binding proteins or their binding to the AP-1 site is likely to be responsible for the observed inhibition of AP-1 transcriptional activity by RME.

DISCUSSION

RME is known to prevent cancer development (5–9). However, the mechanism by which RME exerts its anticancer effect is presently unclear. We have previously examined the effect of RME on the transactivation potential of various retinoid receptors on RAREs and found that RME could modulate the transcriptional activity of retinoid receptors on RAREs (29). Although the biological significance of this effect remains to be seen, the observation that RME could regulate RAR activities led us to study whether RME could modulate the anti-AP-1 effect of retinoid receptors. Our data convincingly demon-
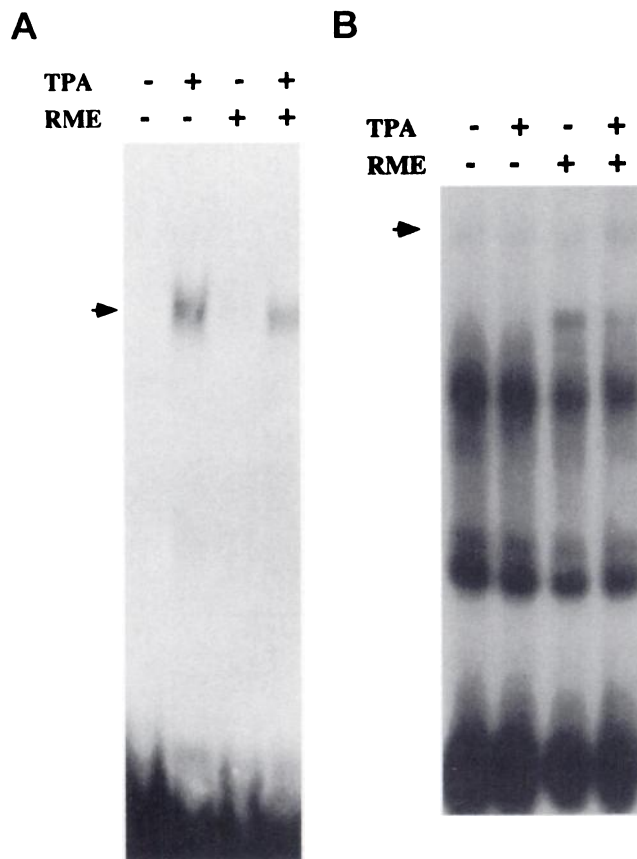

Fig. 6. RME does not inhibit TPA-induced AP-1 binding activity in HeLa cells. Nuclear proteins were prepared from HeLa cells treated with TPA (100 ng/ml) and/or 10^{-6} M RME. A, AP-1 binding of the nuclear proteins was analyzed by gel retardation assay using the ^{32}P-labeled AP-1 binding site (TRE present in the collagenase promoter, TGACTCA) as a probe. *Arrow*, AP-1 binding complex. B, binding of nuclear proteins to SP-1 is shown for control (*arrow*). The result of the gel shift shown is one of two duplicate experiments.

strate that RME can act as an effective anti-AP-1 retinoid. We found that RME could significantly inhibit AP-1 activity induced by TPA and growth factors, and inhibit the transcriptional activity of the nuclear proto-oncogenes c-Jun and c-Fos in HeLa cells (Figs. 1–3) and breast cancer cells (Figs. 4 and 5). Inhibition of AP-1 activity by RME seemed to require RAR-α and/or RXR-α but not RAR-β or RAR-γ, as demonstrated by cotransfection assays in HeLa cells (Fig. 1). Importantly, endogenous RARs in breast cancer cells were able to mediate the anti-AP-1 activity of RME (Figs. 4 and 5). These data are consistent with those of Salbert *et al.* (36) and Soprano *et al.* (37), in which both types of retinoid receptors were required to mediate AP-1 transrepression in hepatocarcinoma cells (HepG2) and ovarian adenocarcinomas, respectively.

The anti-AP-1 activity of retinoid receptors has been considered to be one of the important mechanisms by which retinoids inhibit cell proliferation and cell transformation (14). RARs, in response to RA, could interfere with induction of gene transcription by the AP-1 protein complex (18–20). AP-1 activity can be induced by a wide variety of agents such as mitogens, phorbol esters, stress, and heat shock (20, 30, 36–38). Both insulin and EGF have been implicated in breast tumor growth and are thought to contribute to tumor malignancy (34, 35). AP-1 sites are located in the promoter region of many genes, such as collagenase, stromelysin, and ornithine decarboxylase (20, 30), which play essential roles in cancer cell proliferation and metastasis (39, 40). Inhibition of transcription of these genes may inhibit tumor development and contribute to the anticancer effect of
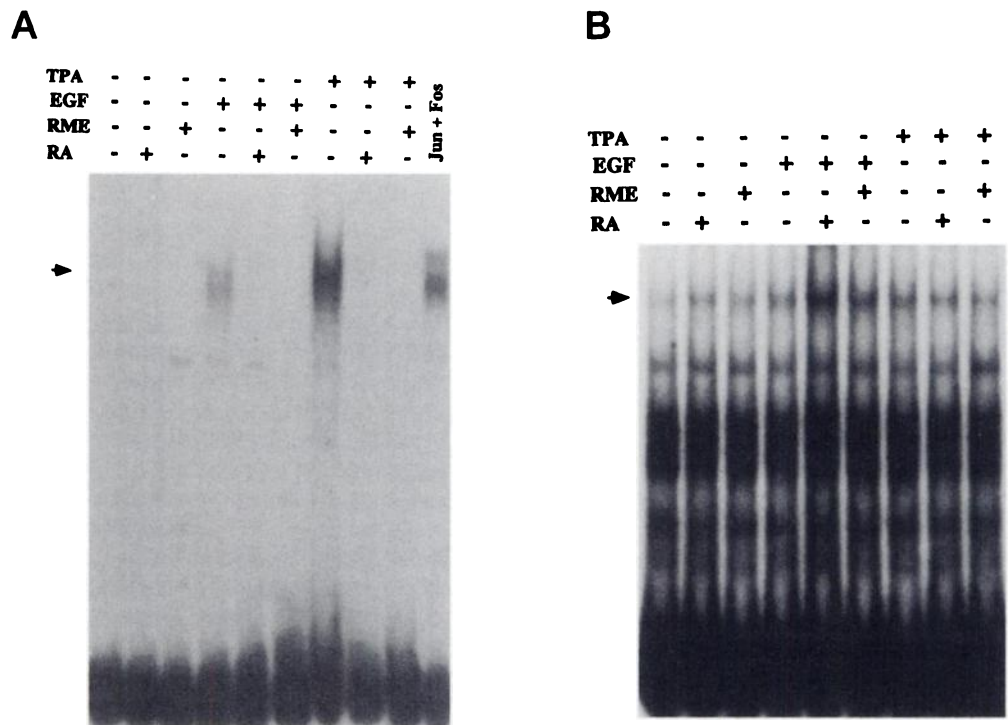
A

B

Fig. 7. Inhibition of TPA- and EGF-induced AP-1 binding activity by RME in ZR-75–1 breast cancer cells. Nuclear proteins were prepared from ZR-75–1 cells treated with TPA (100 ng/ml) or EGF (10 ng/ml) and/or RME (10^{-6} M). *A*, AP-1 binding of the nuclear proteins was analyzed by gel retardation assay using the AP-1 binding site as a probe. *Arrow*, AP-1 binding complex. *B*, binding of nuclear proteins to SP-1 is shown for control (*arrow*). The result of the gel shift shown is one of two duplicate experiments.

retinoids (14). For instance, retinoids that selectively inhibit AP-1 activity were able to inhibit the growth of lung cancer and breast cancer cells (28), and inhibit the TPA-induced transformation and clonal growth of the promotion-sensitive mouse epidermal JB6 cell line (26). Thus, the anti-AP-1 activity could contribute to the chemopreventive effect of retinoids that block the processes of tumor promotion and cell transformation. Our finding that RME can inhibit AP-1 activity induced by a variety of stimuli strongly suggests that the anti-AP-1 effect of RME may contribute to its cancer-preventive effect (5–10). To further study the mechanism by which RME downregulates transcriptional activation of AP-1, we analyzed AP-1 binding of nuclear proteins prepared from HeLa cells (Fig. 6) and ZR-75–1 cells (Fig. 7) treated with TPA or EGF that induces AP-1 activities (30, 41). Our *in vitro* data (Fig. 7) demonstrate that TPA- and EGF-induced AP-1 binding is completely inhibited when breast cancer cells are pretreated with RME, therefore providing an explanation for the *in vivo* repression of AP-1 activity observed in these cells (Figs. 4 and 5).

Modulation of retinoid receptor transactivation activity by retinoids requires their binding to the receptors. *Trans*-RA binds RARs and modulates RAR transactivation activities, whereas 9-*cis*-RA binds both RARs and RXRs and activates their transcriptional function. Inhibition of AP-1 activity by retinoid receptors also requires binding of RA to the receptors (18, 19). In the present study, inhibition of AP-1 activity by RME occurred only when RAR-α and/or RXR-α were expressed (Fig. 1), suggesting that the anti-AP-1 effect of RME is also mediated by retinoid receptors. This is supported by our previous observation that RME could also influence transactivation activities of retinoid receptors on various RAREs (29). RME itself may bind to retinoid receptors and inhibit AP-1 activity *in vivo*. Alternatively, a metabolite of RME may interact with RAR or RXR, thereby modulating the anti-AP-1 activity of the receptors. Previous reports have shown that RARs repress AP-1 binding via protein-protein interactions without competition within the AP-1 binding site (18–20). Inhibition of AP-1 binding by ligand-induced retinoid receptors may be mediated by CBP that interacts with both AP-1

complex and ligand-activated nuclear receptors and acts as a common coactivator of nuclear receptors and AP-1 (22). Our DNA-binding assay data (Fig. 7) suggest that RME-induced retinoid receptors in breast cancer ZR-75–1 cells may inhibit the binding of AP-1 activity in the cells. Alternatively, RME may inhibit the expression of AP-1-binding proteins. Interestingly, RME is able to inhibit the transactivation activity of RAR-α, -β and -γ, whereas it represses AP-1 activity only in the presence of RAR-α, not in the presence of RAR-β and RAR-γ. Thus, binding of RME or its metabolite to RAR-β or RAR-γ may not induce the appropriate conformation required for AP-1 interaction. The finding that RME could not activate receptor transactivation (29) suggests that it represents an anti-AP-1-selective retinoid. This is consistent with the observation that RME is much less toxic than *trans*-RA *in vitro* (7). Research has been focused on the identification of retinoids that selectively inhibit AP-1 activity but do not activate the receptor transactivation function (27, 28). These anti-AP-1-selective retinoids are expected to be more effective anticancer agents and to have reduced side effects related to the inhibition of retinoid receptor transactivation activity.

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