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Retinoic Acid Receptor β Mediates the Growth-Inhibitory Effect of Retinoic Acid by Promoting Apoptosis in Human Breast Cancer Cells

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Retinoids are known to inhibit the growth of hormone-dependent but not that of hormone-independent breast cancer cells. We investigated the involvement of retinoic acid (RA) receptors (RARs) in the differential growth-inhibitory effects of retinoids and the underlying mechanism. Our data demonstrate that induction of RARβ by RA correlates with the growth-inhibitory effect of retinoids. The hormone-independent cells acquired RA sensitivity when the RARβ expression vector was introduced and expressed in the cells. In addition, RA sensitivity of hormone-dependent cells was inhibited by a RARβ-selective antagonist and the expression of RARβ antisense RNA. Introduction of RARα also restored RA sensitivity in hormone-independent cells, but this restoration was accomplished by the induction of endogenous RARβ expression. Furthermore, we show that induction of apoptosis contributes to the growth-inhibitory effect of RARβ. Thus, RARβ can mediate retinoid action in breast cancer cells by promoting apoptosis. Loss of RARβ, therefore, may contribute to the tumorigenicity of human mammary epithelial cells.

Retinoids, the natural and synthetic vitamin A derivatives, are known to regulate a broad range of biological processes, including growth, differentiation, and development (26, 48, 63). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of other human cancers (26, 47, 48, 63). The effects of retinoids are mainly mediated by two classes of nuclear receptors: the retinoic acid (RA) receptors (RARs) (4, 6, 23, 40, 62) and the retinoid X receptors (RXRs) (27, 43, 49, 50, 81). RARs and RXRs are members of the steroid-thyroid hormone receptor superfamily that also includes receptors for estrogen and vitamin D (10, 17, 25, 51, 85). Both types of retinoid receptors are coded for by three distinct genes, α, β, and γ. These receptors display distinct patterns of expression during development and differentiation (26), suggesting that each of them may have specific function. All-*trans* RA and 9-*cis* RA (28, 45), the two known active derivatives of vitamin A, essentially function as hormones by interacting with specific retinoid receptors. All-*trans* RA binds and activates RARs, and 9-*cis* RA is able to bind and activate both RARs and RXRs. RARs and RXRs modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs) (7, 37, 43, 52, 81–83, 86). Some of the target genes are RARs themselves (14, 29, 42, 44, 71), in particular the RARβ gene, for which an RARE (βRARE) that mediates RA-induced RARβ gene expression in many different cell types was identified in its promoter region (14, 29, 71). Autoregulation of the RARβ gene presumably plays a critical role in amplifying the RA response.

Altered nuclear receptor activities are known to be associated with carcinogenesis. In human acute promyelocytic leukemia cells, an abnormal RARα transcript is produced by chromosomal translocation (13, 36). The involvement of RARβ in cancer development was originally suggested by the finding that it was integrated by hepatitis B virus in human hepatoma (12). Recently, it was found that RARβ was not expressed in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, and breast carcinoma (22, 30, 32, 60, 72, 84). Given the fact that retinoids are key players in the regulatory network of cell differentiation and proliferation (26, 48, 63), altered retinoid receptors can result in abnormal cellular differentiation pathways and a loss of their antiproliferating effect, such as anti-AP-1 activity (67, 79). Recently, several studies have reported that retinoids can induce apoptosis in several different cell types (58, 61, 68). Apoptosis, a programmed cell death, is an important physiologic process in normal development and tissue homeostasis and functions as an autonomous suicide pathway that restricts cell numbers (19, 74). Induction of apoptosis by retinoids may represent an important mechanism by which retinoids inhibit cancer cell growth. Alteration of retinoid receptor activity may therefore lead to suppression of apoptosis and result in the pathological accumulation of aberrant cells and diseases involving tumors.

A considerable volume of human and animal data has suggested that retinoids are novel agents for the prevention and treatment of breast cancer (11, 33, 56). In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens (11, 56). In vitro, retinoids have been shown to inhibit the growth of human breast cancer cells (20, 21, 39, 53, 65, 69, 70, 73, 75–77). Growth inhibition of such cells in culture has been observed when retinoids are administered alone or in combination with other agents, such as antiestrogen (20, 39) or interferon (78), with which synergistic effects have been observed. On the basis

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of these results, several clinical trials with retinoids have been carried out (5, 9, 55). Unfortunately, these early clinical trials with patients with advanced breast cancer have not demon strated any significant effect, except that some benefits were observed when retinoids were used together with antiestrogen (5).

The observations that retinoids are effective in the prevention of breast cancer development and that activity is lost in patients with advanced breast cancer suggest that there is a loss of retinoid sensitivity during the progression of a breast tumor. This loss is also supported by in vitro observations that the growth-inhibitory effects of retinoids are mainly seen in hormone-dependent, estrogen receptor (ER)-positive breast cancer cells and that hormone-independent, ER-negative cells are refractory to the retinoid effect (20, 75). How retinoids inhibit the growth of hormone-dependent breast cancer cells and how their inhibitory effect is lost in hormone-independent cells remain largely unclear. In this study, we have determined the expression of RARs in a number of hormone-dependent and -independent breast cancer cell lines in the absence and presence of RA. Our results demonstrated that the expression of the RARβ gene was dramatically induced by RA in hormone-dependent but not in hormone-independent breast cancer cell lines. Induction of RARβ by RA correlated with the growth-inhibitory effect of retinoids in the cell lines investigated. The requirement of RARβ expression for the RA-induced growth inhibition was further demonstrated by restored RA sensitivities in hormone-independent cells after the introduction of RARβ and by diminished RA sensitivities in hormone-dependent cells because of an RARβ-selective antagonist and the expression of RARβ antisense RNA. In addition, our data demonstrated that RARβ could promote apoptosis in breast cancer cells. Thus, the loss of RARβ gene expression could be one of the major factors responsible for the loss of RA sensitivities in breast cancer cells and may contribute to their transformed phenotype.

MATERIALS AND METHODS

Cell culture. Breast cancer cell lines ZR-75-1, T-47D, MB231, BT-20, and MB468 were obtained from the American Type Culture Collection. MCF-7 was obtained from S. Sukumar (Salk Institute, La Jolla, Calif.). ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), MB231, MCF-7, and MB468 cells were grown in Dulbecco modified Eagle medium supplemented with 10% FCS, and BT-20 cells were maintained in minimal essential medium with 10% FCS.

Growth inhibition assay. To study anchorage-dependent growth, cells were seeded at 1,000 to 2,000 cells per well in 96-well plates and treated with various concentrations of retinoids. Media were changed every 48 h. The number of viable cells was determined by measuring their capacity to convert a tetrazolium salt into a blue formazan product with a nonradioactive cell proliferation and cytotoxicity assay kit (Promega, Madison, Wis.) (57). For the anchorage-independent growth assay, 30,000 cells in culture medium containing 10% FCS, 0.3% agar (Difco, Detroit, Mich.), and 10^{-7} M all-*trans* RA in a 60-mm-diameter dish were plated onto an already hardened 0.6% agar underlayer in medium supplemented with 10% FCS. The plates were incubated for 21 days with 5% CO_2. A colony was defined as >40 cells, and colonies with more than 40 cells were counted with a microscope.

RNA preparation and Northern (RNA) blot. For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (66). About 30 μg of total RNAs from different cell lines was fractionated on a 1% agarose gel, transferred to nylon filters, and probed with the ^{32}P-labeled ligand-binding domain of RAR cDNAs as previously described (84). To determine that equal amounts of RNA were used, the filters were also probed with rRNA L32 cDNA.

Preparation of nuclear extracts and gel retardation assays. Nuclear extracts were prepared essentially according to the method previously described (41). Briefly, cells growing to about 90% confluence were washed with cold phosphate-buffered saline (PBS) and scraped into PBS with a rubber policeman. Cells were pelleted by low-speed centrifugation and then resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$, and 2 mM $MgCl_2$. After they were pelleted, the cells were lysed in the buffer containing 1% Nonidet P-40 by 10 to 15 strokes with an ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at 2,000 × *g* and washed once with a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol. Nuclear proteins were extracted with a high-salt buffer containing 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 dithiothreitol. All the buffers used for the procedure contained protease inhibitors, i.e., 100 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of leupeptin per ml, and 1 μg of aprotinin per ml. When it was necessary, nuclear extracts were concentrated with a Centricon 10 microconcentrator (Millipore). Small aliquots of nuclear proteins were immediately frozen and kept at −80°C until use. To study βRARE binding, nuclear extracts were prepared from different breast cancer cell lines treated or not treated with 10^{-6} M all-*trans* RA. Nuclear extracts (5 μg) from different breast cancer cells were analyzed by gel retardation assays for their βRARE binding activity, with ^{32}P-labeled βRARE being used as a probe as described previously (41). βRARE used in the experiments is the direct repeat of the RARE present in the RARβ promoter (AGGGTTCAGGCAAAGTTCAC). Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

Transient transfection and CAT assay. To measure the transcriptional activation of βRARE in breast cancer cell lines, βRARE linked with the chloramphenicol acetyltransferase (CAT) gene (βRARE-tk-CAT) was used as a reporter gene to determine the RA response in hormone-dependent and -independent human breast cancer cell lines. βRARE-tk-CAT (2.0 μg) and 3.0 μg of β-galactosidase expression vector (pCH 110; Pharmacia) were transiently transfected into cells by the calcium phosphate precipitation method (41). Cells were grown in the presence or absence of 10^{-7} M all-*trans* RA. Transfection efficiency was normalized by β-galactosidase activity. The gathered data were shown as the means of three separate experiments.

Stable transfection. To construct RARβ and RARα expression vectors, cDNA for the RARβ or RARα gene was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.). To construct the RARβ antisense expression vector, cDNA for the RARβ gene was cloned into the pRc/CMV expression vector in an antisense orientation. The resulting recombinant constructs were then stably transfected into breast cancer cells by the calcium phosphate precipitation method and screened with 400 μg of G418 (Gibco BRL, Grand Island, N.Y.). The integration and expression of exogenous RARβ and RARα cDNA were determined by Southern blotting and Northern blotting, respectively.

Apoptosis analysis. For morphological analysis (34), cells treated with 10^{-6} M all-*trans* RA and untreated cells were trypsinized and washed with PBS. After fixation with 3.7% paraformaldehyde followed by acid-alcohol treatment, the cells were stained with propidium iodine (50 μg/ml) containing 100 μg of DNase-free RNase A per ml to visualize the nuclei. Stained cells were examined with a Zeiss LSM 410 confocal laser-scanning microscope. Overlays of cells were made with confocal sections at increments of 1 μm. Apoptotic nuclei were condensed and were more brightly stained than nonapoptotic ones. For terminal deoxynucleotidyl transferase (TdT) assays (24), the cells were treated or not treated with 10^{-6} M all-*trans* RA. After 24 h, the cells were trypsinized, washed with PBS, and fixed in 1% formaldehyde in PBS (pH 7.4). After being washed with PBS, the cells were resuspended in 70% ice-cold ethanol and immediately stored overnight at −20°C. The cells were then labeled with biotin-16–dUTP by the terminal transferase method and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescence-labeled cells were analyzed with a FACStar Plus. Representative histograms were made. For enzyme-linked immunosorbent assays (ELISAs), cells from breast cancer cell lines MB231, MB231/RARβ3, ZR-75-1, and ZR-75-1/A-RARβ10 were split at the same time and treated with 10^{-6} M all-*trans* RA for 12, 24, and 48 h. When antagonists (5×10^{-7} M) were used, they were incubated or not incubated with 10^{-7} M all-*trans* RA for 24 h. Treated cells were harvested at the same time. DNA fragmentation was measured with the cell death detection ELISA kit (Boehringer). About 2×10^4 cells were assayed for DNA fragmentation by following the manufacturer's protocol. The results were expressed relative to those for controls that did not receive RA treatment.

RESULTS

Activation of RARs and not RXRs is responsible for RA-induced growth inhibition in breast cancer cells. Retinoids are known to inhibit the growth of breast cancer cells. However, how the growth-inhibitory effect of retinoids is mediated is largely unknown. To establish the involvement of RAR and RXR in RA-induced growth inhibition in hormone-dependent breast cancer cells, we used retinoids selective for RXR homodimers and RXR-RAR heterodimers. Ch55 (35) and (all-E)-UAB8 (1), which specifically bind RARs and activate RXR-RAR heterodimers, displayed degrees of growth inhibition similar to that observed with all-*trans* RA in ZR-75-1 and T-47D cells, while (9Z)-UAB8 (1), which specifically activates RXR homodimers, did not show clear growth-inhibitory effects (Fig. 1). These data suggest that activation of RARs is mainly responsible for

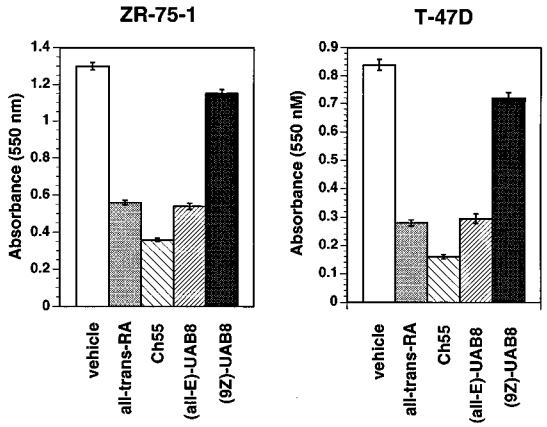


FIG. 1. Activation of RARs but not RXRs is required for RA-induced growth inhibition in breast cancer cell lines. The effects of RAR-RXR heterodimer [Ch55 and (all-E)-UAB8] and RXR homodimer [(9Z)-UAB8] specific activators on the growth of hormone-dependent breast cancer cells (ZR-75-1 and T-47D) are shown in graphic form. The effect of all-*trans* RA is shown for purposes of comparison. Cells were seeded at 1,000 cells per well and treated with 10^{-7} M retinoids for 10 days. The results were expressed as the A_{550} of MTT-derived formazan developed by cells treated with control solvent. All data shown are representative of three independent experiments. Error bars indicate standard deviations.

the RA-induced growth inhibition of ZR-75-1 and T-47D breast cancer cells, consistent with a previous observation (69).

Induction of RARβ by RA correlates with the growth-inhibitory effect of RA. To determine which RAR subtype is involved in RA-induced growth inhibition, we investigated the expression of three types of RARs (α, β, and γ) in a number of human breast cancer cell lines, including hormone-dependent (T-47D, ZR-75-1, and MCF-7) and hormone-independent (MB-468, BT-20, and MB231) lines. In a result similar to those of previous observations (64, 75), transcript for RARγ was detected in all of the cell lines at similar expression levels (Fig. 2a). RARα transcripts were also present in all of the cell lines. However, relatively low levels of RAR expression were found in two hormone-independent cell lines (MB231 and MB-468) (Fig. 2b). All of these cell lines did not exhibit detectable levels of RARβ mRNA under the conditions used (Fig. 2c). Since the expression of RARs could be regulated by RA because of the presence of RAREs in their promoter regions (14, 29, 42, 44, 71), we analyzed the expression of RARs in the presence of RA in these cell lines. Treatment of these cells with 10^{-6} M all-*trans* RA for 36 h did not show any effect on the expression levels of RARα and RARγ (Fig. 2a and b). However, the expression of RARβ was strongly enhanced by all-*trans* RA in the hormone-dependent cell lines (Fig. 2c). Surprisingly, all-*trans* RA failed to induce RARβ in hormone-independent cell lines. When the growth-inhibitory effects of all-*trans* RA and 9-*cis* RA were examined, both RAs showed strong growth inhibition with hormone-dependent cell lines, while they had little effect on hormone-independent lines (Fig. 2d). Thus, the induction of RARβ gene expression by all-*trans* RA correlates with all-*trans* RA-induced growth inhibition, suggesting that RARβ may mediate the differential growth-inhibitory effects of RA in breast cancer cells.

Abnormal transcriptional regulation of βRARE in hormone-independent breast cancer cells. All-*trans* RA-induced RARβ expression is mediated by the βRARE present in the RARβ promoter (14, 29, 71). The loss of the all-*trans* RA effect in inducing RARβ gene expression in hormone-independent human breast cancer cell lines indicates that the regulation of RARβ expression by RA is disturbed in these cells. To further examine the impaired RA response, a CAT reporter construct containing βRARE linked with a thymidine kinase promoter (βRARE-tk-CAT) (29) was used as a reporter to determine the degrees of RA response in both hormone-dependent and -independent cancer cell lines by transient transfection assays. When this reporter was transfected into hormone-dependent cells (T-47D and ZR-75-1), a strong induction of CAT activity in response to all-*trans* RA was observed (Fig. 3a). In contrast, only a slight induction of CAT gene expression was seen in hormone-independent cells (MB231 and MB468). These results are consistent with those of a previous observation (75) and suggest that the loss of RARβ expression in hormone-independent breast cancer cells may be due to an abnormal transcriptional regulation of βRARE. To investigate whether the loss of the βRARE activity is due to altered βRARE binding, nuclear proteins from hormone-dependent and -independent breast cancer cells that were treated with all-*trans* RA or untreated were prepared and analyzed by gel retardation for their binding to βRARE. As can be seen in Fig. 3b, strong DNA binding complexes were formed when nuclear proteins prepared from ZR-75-1 or T-47D cells were used. The binding of the complexes was much stronger when nuclear proteins were prepared from cells treated with all-*trans* RA. However, complexes were hardly seen when nuclear proteins prepared from hormone-independent cells (MB468 and MB231) that were treated with all-*trans* RA or untreated were used. Thus, altered βRARE binding activity may be responsible for the loss of all-*trans* RA-induced βRARE transcriptional activation in the hormone-independent cells.

Recovery of RA sensitivity in hormone-independent breast cancer cells by RARβ expression. The data outlined above suggest that induction of RARβ by all-*trans* RA may be responsible for the RA-induced growth inhibition in hormone-dependent breast cancer cells and that the loss of RA sensitivity in hormone-independent breast cancer cells may be due to a lack of or low levels of RARβ in these cells. To directly test this, cDNA for the RARβ gene was cloned into the pRc/CMV vector so that the expression of the RARβ gene is under the control of the cytomegalovirus promoter. The vector also contains a neomycin resistance gene that allows transfected cells to grow in the presence of G418. pRc/CMV-RARβ was transfected into hormone-independent breast cancer cells (MB231). Six neomycin resistance MB231 clones that carried the exogenous RARβ gene, as revealed by Southern blot analysis (data not shown), were selected. Among these clones, MB231/RARβ2 and MB231/RARβ3 expressed exogenous RARβ gene transcripts (Fig. 4a), as judged by their sizes, which were smaller than that of the endogenous RARβ transcript observed in RA-treated ZR-75-1 cells (data not shown). The rest of the clones and cells transfected with pRc/CMV empty vector did not show any RARβ transcript.

To determine the effect of the introduced RARβ, the growth of MB231/RARβ2, MB231/RARβ3, MB231/RARβ9, and cells transfected with empty vector was measured in the presence or absence of either all-*trans* RA or 9-*cis* RA by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 4b). For cells in the presence of RA, we observed a strong growth inhibition of RARβ-transfected cells. Treatment with 10^{-6} M RA resulted in an about 50% inhibition of MB231/RARβ2 cell growth. Under the same conditions, about 40% inhibition was seen in MB231/RARβ3 cells. In contrast, MB231 cells transfected with empty vector or parental MB231 cells did not show any response to RA. The fact that cells (MB231/RARβ9) transfected with pRc/CMV-RARβ vector but failing to express RARβ (Fig. 4a) did not show any response to RA (Fig. 4b) suggests that the growth-inhibitory

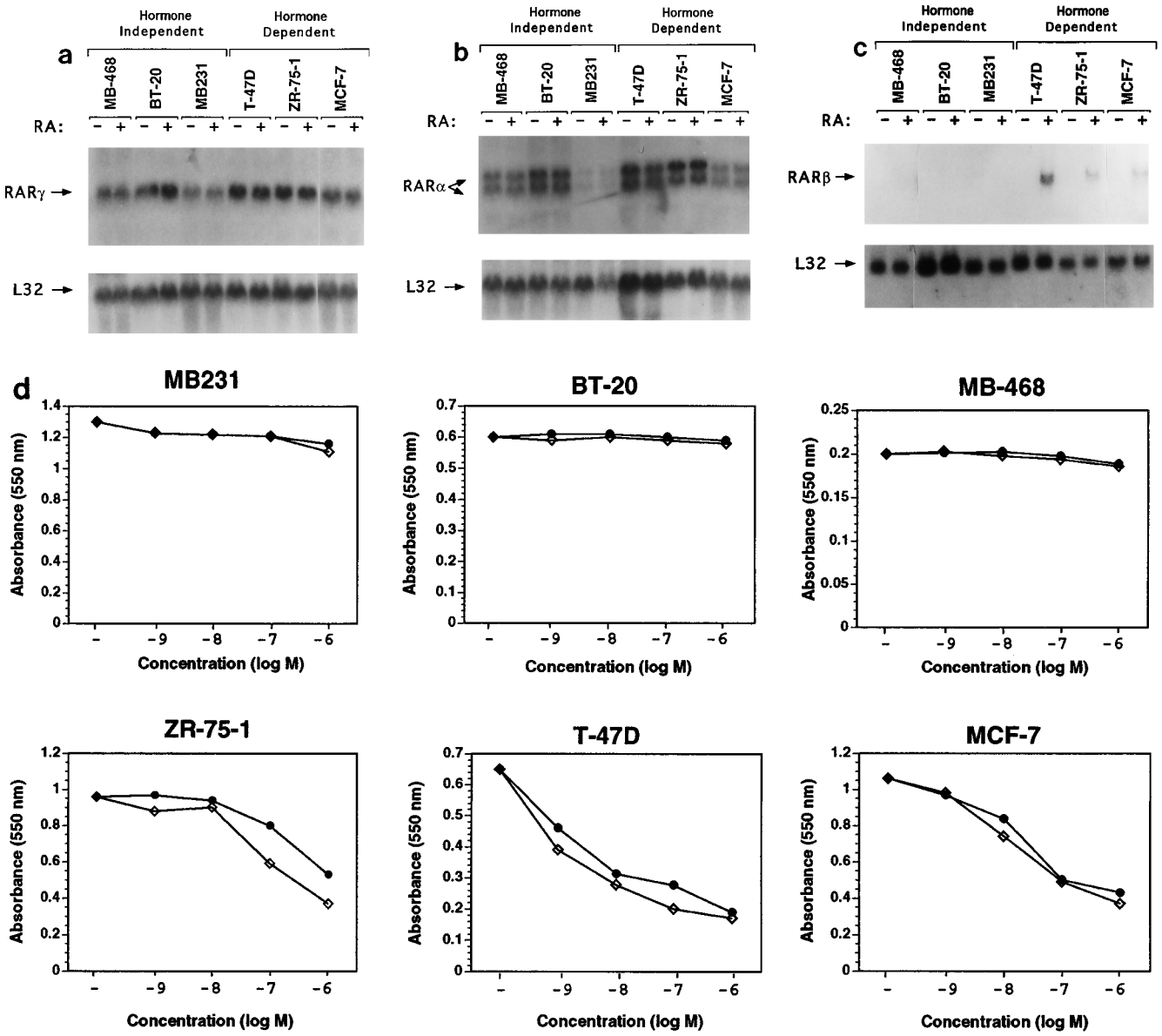


FIG. 2. Expression of retinoid receptors and growth-inhibitory effect of RA in human breast cancer cells. Expression of RARγ (a), -α (b), and -β (c) in hormone-dependent and -independent human breast cancer cell lines is shown. The expression of RAR genes was determined by Northern blot analysis, with total RNA (about 30 μg) prepared from different breast cancer cell lines being used. To determine the effect of RA, cells were treated with 10^{-6} M all-*trans* RA for 36 h before RNA preparation. (d) Effect of all-*trans* RA (solid circles) and 9-*cis* RA (open diamonds) on the growth of hormone-dependent and -independent breast cancer cells. A total of 2,000 cells per well were seeded and treated with various concentrations of RA for 7 days. Growth inhibition was performed as described in the legend to Fig. 1.

effect of RA is mediated by the RARβ product. To further characterize the effect of the transfected RARβ gene, RARβ-transfected cells were analyzed for anchorage-independent growth in soft agar. As can be seen from Fig. 5, the growth of transfectant cells that expressed RARβ (MB231/RARβ2 and MB231/RARβ3) in soft agar was dramatically inhibited by all-*trans* RA, whereas the growth of parental MB231 cells was not affected. Together, these data demonstrate that the expression of RARβ can restore RA sensitivity in hormone-independent breast cancer cells.

Induction of RARβ by RARα is responsible for the recovery of RA sensitivity in hormone-independent breast cancer cells. The expression of RARα is relatively low in some of the hormone-independent cell lines, such as MB231 and MB-468 (Fig. 2b). This low expression has been previously suggested to account for RA resistance in hormone-independent cells (69, 70). We then investigated whether the expression of RARα could restore RA sensitivity in hormone-independent cells. RARα cDNA was cloned into the pRc/CMV vector, and the resulting expression plasmid was stably transfected into MB231 cells. Two clones (MB231/RARα1 and MB231/RARα2) that expressed introduced RARα gene (data not shown) showed a strong RA growth-inhibitory effect in a concentration-dependent manner, while RARα had little effect on the growth of parental MB231 cells (Fig. 6a). Since all-*trans* RA is known to activate βRARE (14, 29, 71), we therefore investigated whether expression of introduced RARα could result in the induction of endogenous RARβ. As can be seen in Fig. 6b, the

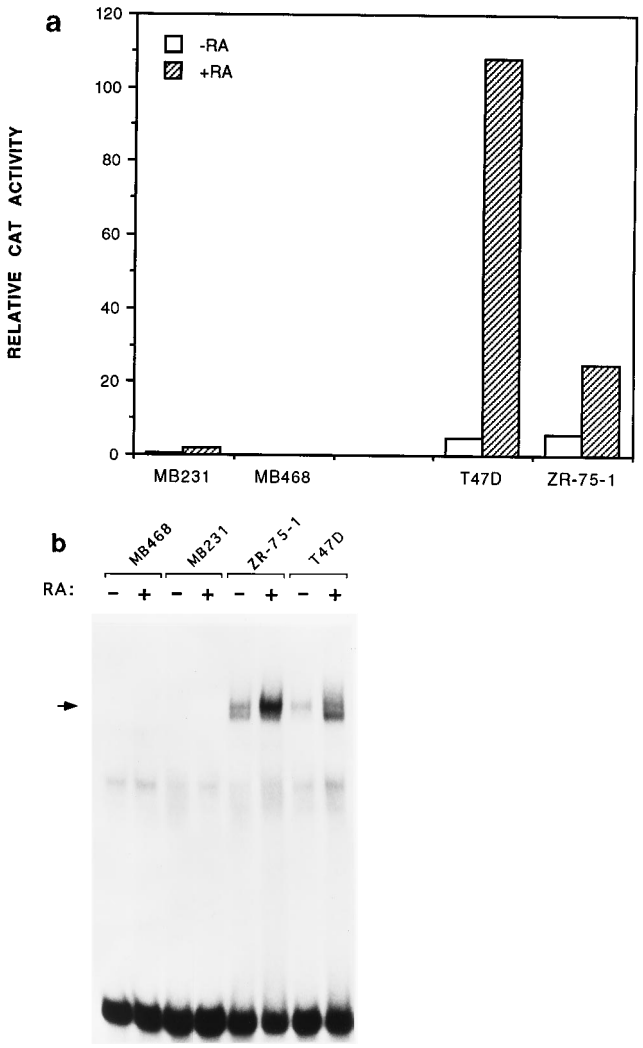


FIG. 3. Transcriptional activity and binding of βRARE in human breast cancer cell lines. (a) Transcriptional activation of βRARE in hormone-dependent and -independent human breast cancer cell lines. Transient transfection assays were used to determine transcriptional activation of βRARE in various human breast cancer cell lines. (b) βRARE binding of nuclear proteins prepared from hormone-dependent and -independent human breast cancer cell lines. The arrow indicates the specific binding complex present in breast cancer cells.

expression of endogenous RARβ was significantly enhanced in several different clones that expressed exogenous RARα when these cells were treated with 10^{-6} M all-*trans* RA. Thus, activation of RARα can result in the induction of RARβ in breast cancer cells. Furthermore, Ro 41-5253 (2), an RARα-selective antagonist, strongly inhibited the induction of RARβ by all-*trans* RA in T-47D and ZR-75-1 cells (Fig. 6c). Ro 41-5253 but not LE135, an RARβ-selective antagonist (18, 46), was also able to inhibit all-*trans* RA-induced RARβ expression in MB231/RARα2 (Fig. 6c). Together, the growth-inhibitory effect of RARα is likely due to its induction of the endogenous RARβ through activation of βRARE.

RARβ is essential for RA-induced growth inhibition in hormone-dependent cells. To investigate whether the induction of RARβ by RA can mediate the RA-induced growth inhibition in hormone-dependent cells, we used an RARβ-selective antagonist (LE135) (18, 46) to suppress RARβ activity in the cells. This retinoid can specifically inhibit RARβ- but not RARα- or RARγ-mediated activation of target genes (46). As can be seen from Fig. 7a, a concentration-dependent reduction in RA-induced growth inhibition was observed in both ZR-75-1 and T-47D cells when LE135 was added to cells together with 10^{-7} M all-*trans* RA. In the presence of 10^{-6} M LE135, all-*trans* RA-induced growth inhibition in ZR-75-1 cells was reduced from about 50 to 20%. A similar degree of effect was seen with T-47D cells. These data suggest that activation of RARβ is mainly responsible for RA-induced growth inhibition in hormone-dependent breast cancer cells. To further support this conclusion, we stably transfected an RARβ antisense cDNA cloned into pRc/CMV into ZR-75-1 cells. Expression of antisense mRNA is known to reduce the level of its target mRNA by hybridization, which results in the degradation of the double-stranded RNA (8). As can be seen from Fig. 7b, clone A-RARβ5 did not show detectable expression of RARβ antisense RNA. The expression of endogenous RARβ in this clone is highly induced by RA in a manner similar to that for parental cells as determined by Northern blot analysis (Fig. 7b and data not shown). In contrast, clone A-RARβ10, which expressed RARβ antisense RNA, failed to express endogenous RARβ under all-*trans* RA treatment (Fig. 7b). When the growth-inhibitory effect of all-*trans* RA was examined in two clones that expressed RARβ antisense RNA (A-RARβ10 and A-RARβ25) (Fig. 7b and data not shown), we observed reduced all-*trans* RA-induced growth inhibition in these clones (Fig. 7c). In contrast, the growth of A-RARβ5 remained strongly inhibitable by all-*trans* RA (Fig. 7c). These results, therefore, demonstrate that expression of RARβ is essential for RA-induced growth inhibition in hormone-dependent breast cancer cells.

RA-activated RARβ promotes apoptosis in breast cancer cells. When RARβ was expressed in MB231 cells, we noticed a morphological change in the cells. MB231 cells, when seeded at low density, were elongated (Fig. 8a). However, MB231 cells that expressed RARβ were relatively round and became shrunken. Such a morphological change was observed even in the absence of RA, probably because of the constitutive high level of RARβ expression and the presence of residual amounts of retinoids in the serum. The ability of the cells that expressed RARβ to survive on culture dishes was quite different from that of wild-type cells. After the continuation of culture at low density, a large portion of cells eventually died, particularly in the presence of all-*trans* RA, as assayed by the trypan blue dye exclusion method (data not shown). To investigate whether the loss of survivability of the cells is due to apoptosis, we examined the nuclear morphology of MB231 cells that expressed RARβ. When the nuclei of these cells were stained with propidium iodine and examined by confocal fluorescence microscopy, we found that many of the RA-treated MB231/RARβ3 cells were smaller and contained condensed and fragmented nuclei with brightly stained chromatin, i.e., morphological changes typical of apoptosis (74) (Fig. 8b). RA caused similar nucleus morphological alterations in the all-*trans* RA-sensitive lines ZR-75-1, MCF-7, and T-47D (Fig. 8c, d, and e) but not in the all-*trans* RA-resistant lines MB231, MB-468, and BT-20 (data not shown).

To further study RA-induced apoptosis in breast cancer cells, we carried out TdT assays with flow cytometric analysis to study DNA fragmentation. As can be seen in Fig. 8f, the stable expression of RARβ in MB231 cells (MB231/RARβ3) resulted in significant amounts of TdT-labeled cells when they were treated with 10^{-6} M all-*trans* RA for 24 h. Treatment of MB231 cells with all-*trans* RA did not show a clear increase of TdT-labeled cells. In ZR-75-1 cells, a marked increase in TdT-labeled cells was observed in response to all-*trans* RA. However, the TdT labeling was significantly inhibited when RARβ

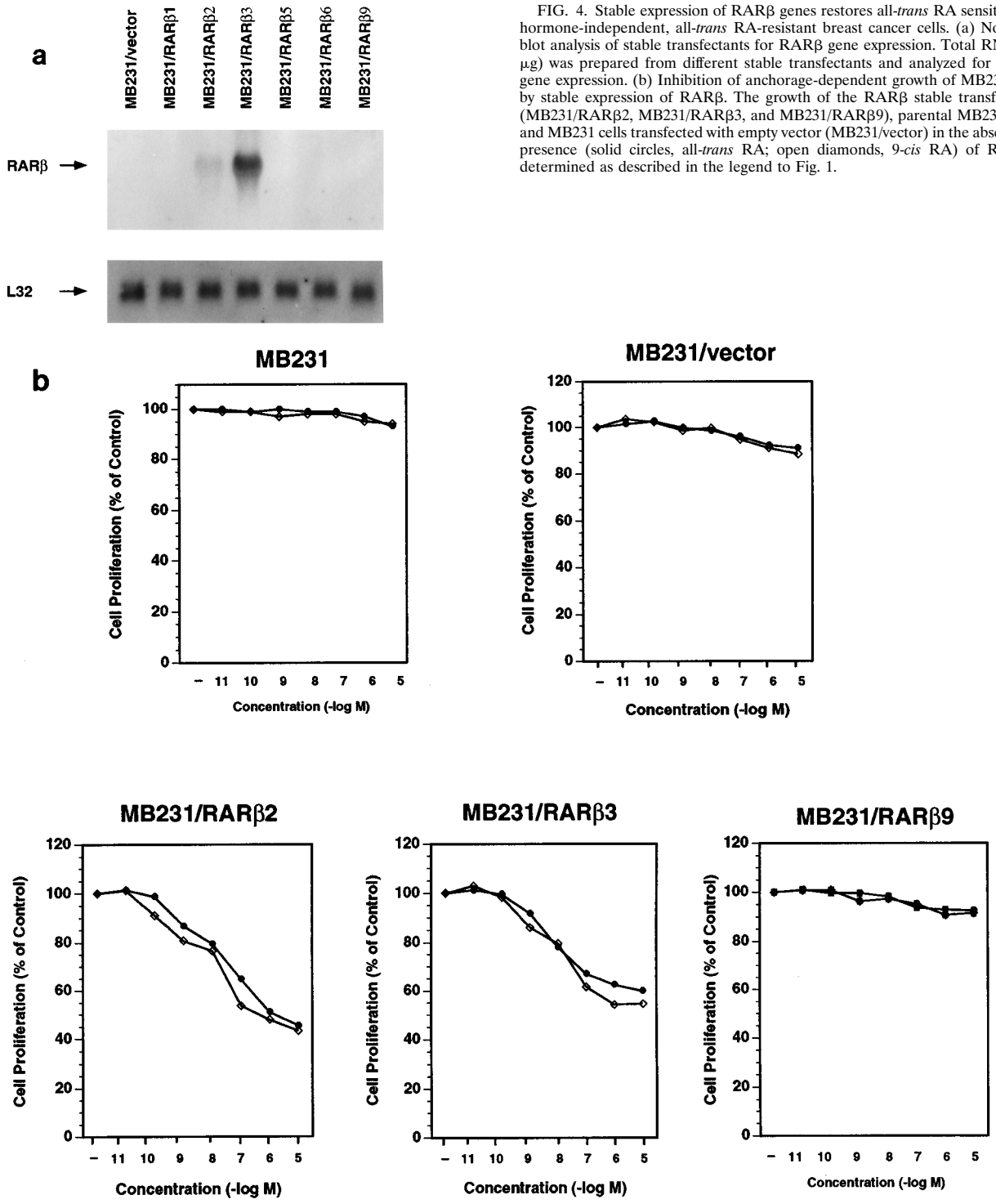


FIG. 4. Stable expression of RARβ genes restores all-*trans* RA sensitivity in hormone-independent, all-*trans* RA-resistant breast cancer cells. (a) Northern blot analysis of stable transfectants for RARβ gene expression. Total RNA (30 μg) was prepared from different stable transfectants and analyzed for RARβ gene expression. (b) Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RARβ. The growth of the RARβ stable transfectants (MB231/RARβ2, MB231/RARβ3, and MB231/RARβ9), parental MB231 cells, and MB231 cells transfected with empty vector (MB231/vector) in the absence or presence (solid circles, all-*trans* RA; open diamonds, 9-*cis* RA) of RA was determined as described in the legend to Fig. 1.

antisense RNA was expressed (A-RARβ10). Similar results were obtained by another assay (Fig. 8g), which is based on the sandwich enzyme immunoassay principle to determine cytoplasmic histone-associated DNA fragments in apoptotic cells. This study also revealed that DNA fragmentation in ZR-75-1 and MB231/RARβ3 occurred as early as 12 h after the cells were exposed to all-*trans* RA. Furthermore, LE135 but not Ro 41-5253 was able to inhibit all-*trans* RA-induced apoptosis in MB231/RARβ3. Together, these results clearly demonstrate that all-*trans* RA can induce apoptosis in breast cancer cells and that all-*trans* RA-induced apoptosis is mediated by RARβ in the cells.

DISCUSSION

Retinoids are effective growth inhibitors of breast cancer cells. However, inhibition of growth is often observed in hor-

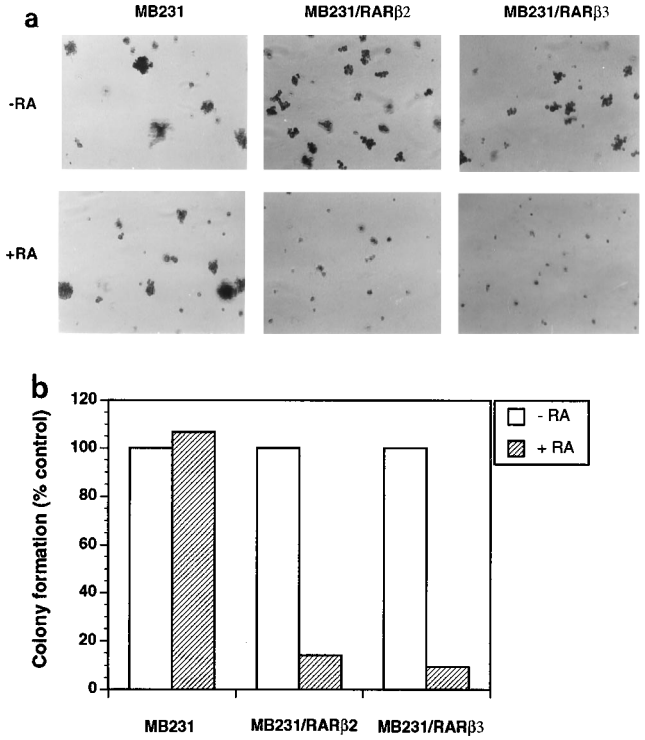


FIG. 5. Inhibition of anchorage-independent growth of MB231 cells by RARβ gene expression. (a) Photograph of colonies formed by parental MB231, MB231/RARβ2, and MB231/RARβ3 cells. (b) Quantitation of colonies formed by parental MB231, MB231/RARβ2, and MB231/RARβ3 cells. Colonies formed by MB231/RARβ2, MB231/RARβ3, and parental MB231 cells in the presence or absence of all-*trans* RA were scored and expressed as percentages of the number of colonies formed by cells treated with control solvent.

mone-dependent but not in hormone-independent breast cancer cells (20, 75). In this study, we have demonstrated that the expression of the RARβ gene is critical for RA-induced growth inhibition. By using receptor-selective retinoids, we first show that activation of RXRs is not involved in the cell lines (ZR-75-1 and T-47D) studied (Fig. 1). Retinoids that activate RXR and induce RXR homodimer formation did not show a clear effect on the growth of two different hormone-dependent breast cancer cell lines (ZR-75-1 and T-47D), while retinoids that activate RARs were as effective as all-*trans* RA. These data are consistent with results obtained from a previous study that used different RXR-selective retinoids in MCF-7 and other breast cancer cell lines (69). Thus, selective activation of the RXR homodimer pathway may not contribute substantially to RA-induced growth inhibition in the breast cancer cells analyzed, and activation of the RAR pathway may be critical. Previously, several studies to elucidate the role of RARs in the differential growth-inhibitory effects of RA in hormone-dependent and -independent breast cancer cell lines were carried out (64, 75). These studies and the present study (Fig. 2) showed comparable expression levels of RARγ mRNA in all the cell lines, regardless of the ER status, and no marked expression level changes under all-*trans* RA treatment, indicating that RARγ is unlikely to be involved in the differential growth-inhibitory function of all-*trans* RA. However, a recent study suggests that RARγ may function to mediate the synergistic growth-inhibitory effect of RA and interferon on breast cancer cells (78). In the case of RARα, Roman et al. (64) found that it was expressed in all cell lines, with higher levels being found in hormone-dependent cell lines than in independent lines. In our study, two RAR transcripts were observed in all cell lines investigated. However, they were less abundant in MB231 and MB468 cell lines (Fig. 2) but were highly expressed in another hormone-independent line (BT-20). Although these results suggest that RARα may be involved, the variations in the expression levels of RARα (reference 64 and this study) cannot satisfactorily explain the dramatic differences in the sensitivities of different breast cancer cell lines to RA. So far, the expression of RARβ has been investigated by several stud-

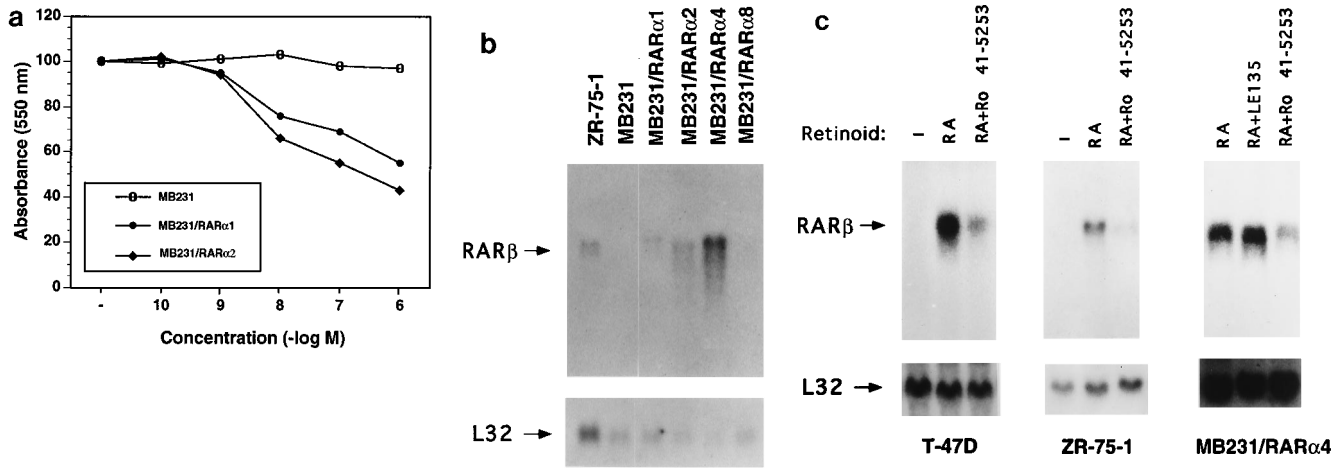


FIG. 6. Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RARα. (a) The growth of RARα stable transfectants (MB231/RARα1 and MB231/RARα2) and parental MB231 cells was analyzed in the presence of various concentrations of all-*trans* RA as described in the legend to Fig. 1. (b) Expression of endogenous RARβ gene in RARα stable transfectants. Northern blotting was used to analyze the expression of the endogenous RARβ gene in MB231 cells that stably expressed RARα in the presence of 10^{-6} M all-*trans* RA. For purposes of comparison, the expression of RARβ in ZR-75-1 cells treated with all-*trans* RA is shown. The expression of the L32 gene was used as a control. (c) Inhibition of RA-induced RARβ gene expression by an RARα-selective antagonist. The expression of the RARβ gene in ZR-75-1 and T-47D cells in the presence of 10^{-7} M all-*trans* RA together with or without 10^{-6} M RARα-selective antagonist (Ro 41-5253) was analyzed by Northern blotting as described in the legend to Fig. 2. When MB231/RARα4 cells were analyzed, 5×10^{-8} M all-*trans* RA and 10^{-7} M Ro 41-5253 and 10^{-7} M LE135 were used.

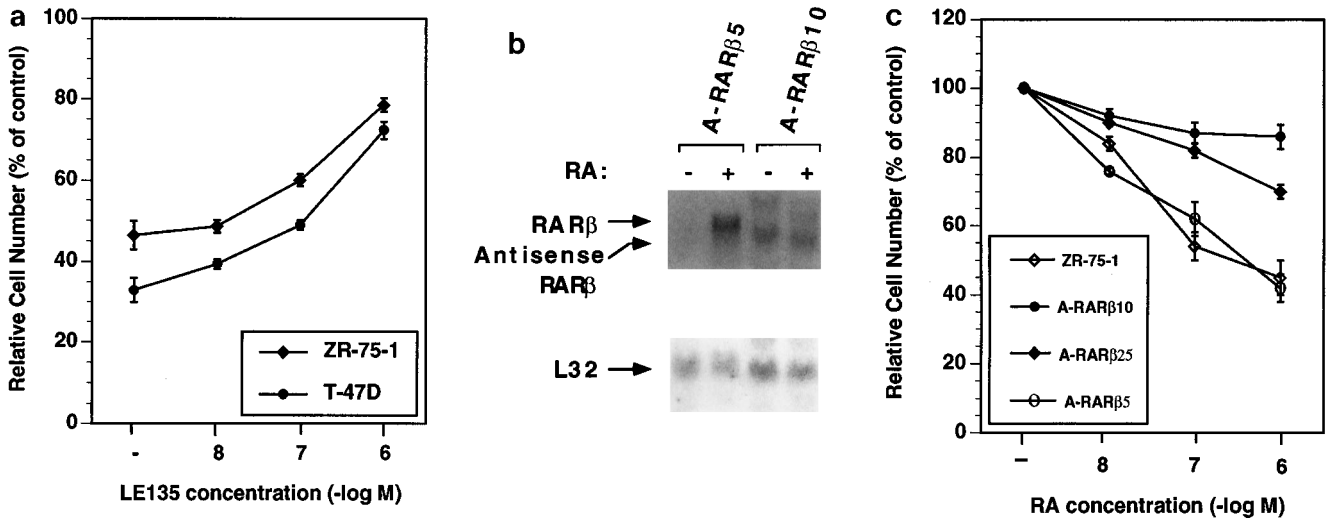


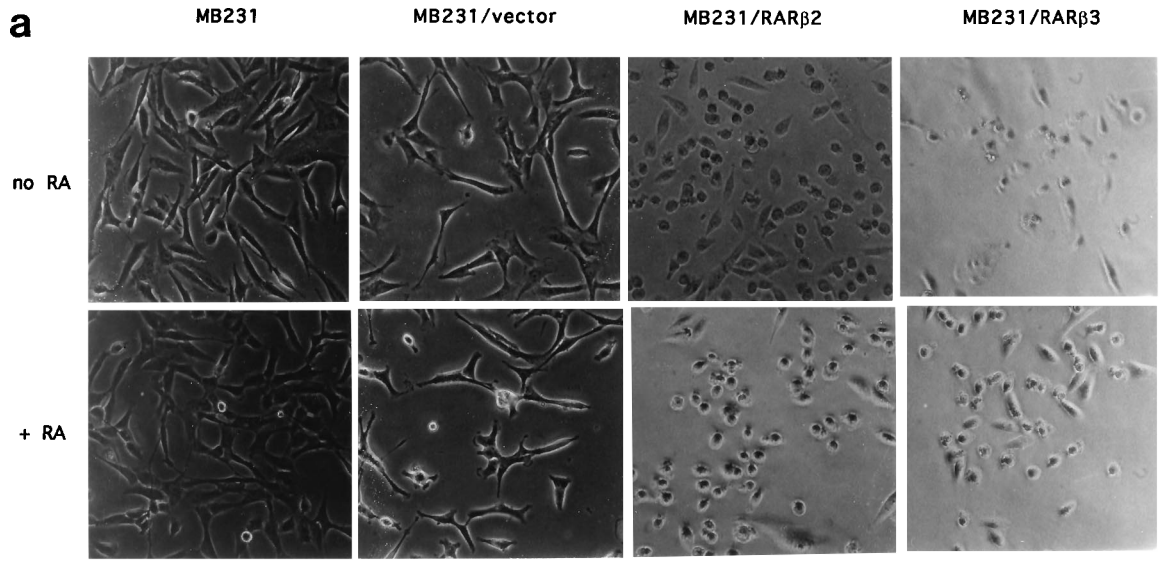
FIG. 7. Inhibition of RARβ activity decreases RA sensitivity in hormone-dependent breast cancer cells. (a) RARβ-selective antagonist decreases the growth-inhibitory effect of all-*trans* RA in ZR-75-1 and T-47D cells. ZR-75-1 and T-47D cells were treated with or without 10^{-7} M all-*trans* RA in the presence or absence of an RARβ-selective antagonist (LE135 K_i for RARα, 1.5×10^{-6} M; LE135 K_i for RARβ, 4.3×10^{-8} M) for 10 days, and the numbers of the cells were analyzed by the MTT assay as described in the legend to Fig. 1. (b) Expression of RARβ in ZR-75-1 cells transfected with RARβ antisense cDNA. The expression of endogenous RARβ in two stable clones (A-RARβ5 and A-RARβ10) was determined by Northern blotting. A-RARβ10 expressed introduced RARβ antisense RNA, while no clear expression was seen with A-RARβ5. (c) Expression of RARβ antisense RNA decreases RA sensitivity in hormone-dependent breast cancer cells. The effect of all-*trans* RA on the growth of ZR-75-1 cells and ZR-75-1 cells that stably expressed RARβ antisense RNA (A-RARβ10 and A-RARβ25) was analyzed by MTT assay as described in the legend to Fig. 1. Error bars show standard deviations.

ies (64, 72, 75). van der Burg et al. (75) observed high levels of RARβ in two of the three hormone-dependent lines (ZR-75-1 and T-47D) but not in independent lines, except for Hs578T. In contrast, RARβ was expressed in all independent cell lines analyzed by Roman et al. (64), while it was not detected in dependent lines, including T-47D and MCF-7, or was expressed at a low level in other dependent lines. In another study (72), RARβ transcript in MCF-7 and ZR-75-1 cells could only be detected when poly(A)$^+$ RNA was used. These different results may be due to the variability of the cell lines used but are more likely to be due to the different culture conditions used, since the expression of RARβ is very sensitive to RA regulation because of the presence of βRARE in its promoter (14, 29, 71). To clearly establish the role of RARβ, we have examined the expression of RARβ in several hormone-dependent and -independent cell lines either in the presence or in the absence of all-*trans* RA (Fig. 2). In the absence of all-*trans* RA, all of these cell lines did not exhibit detectable RARβ transcript. However, in the presence of RA, the expression of RARβ was strongly enhanced in hormone-dependent cell lines (ZR-75-1, T-47D, and MCF-7) but not in independent cell lines (MB231, BT-20, and MB468). The correlation of all-*trans* RA-induced RARβ expression with all-*trans* RA-induced growth inhibition in these cells (Fig. 2) implies that RARβ is required for all-*trans* RA-induced growth inhibition in breast cancer cell lines.

The role of RARβ in mediating all-*trans* RA-induced growth inhibition is further demonstrated by our stable transfection studies. Hormone-independent MB231 cells that are devoid of RARβ did not show a growth response to all-*trans* RA (Fig. 2). However, when the RARβ gene was introduced and expressed in the cells, the inhibitory effects of all-*trans* RA on the anchorage-dependent and -independent growth of the cells were observed (Fig. 4 and 5). In contrast, hormone-dependent ZR-75-1 cells that stably expressed RARβ antisense RNA showed a strong reduced all-*trans* RA sensitivity (Fig. 7). In addition, when we used an RARβ-selective antagonist (LE135) (46) together with all-*trans* RA, we found that it could significantly prevent all-*trans* RA-induced growth inhibition of ZR-75-1 and T-47D cells (Fig. 7). Together, these data clearly demonstrate that RARβ can mediate the all-*trans* RA-induced growth inhibition in breast cancer cells. This conclusion is supported by a recent study showing that senescence of normal human mammary epithelial cells resulted in increased RARβ mRNA expression (72).

Previous studies (64, 70, 75) and this study (Fig. 2) show that RARα transcripts are not expressed or are expressed at relatively low levels in certain hormone-independent cell lines, suggesting that RARα may also participate in mediating the growth inhibition of all-*trans* RA. Indeed, we found that RARα could restore all-*trans* RA sensitivity when it was stably expressed in MB231 cells (Fig. 6), which is a result similar to that of a previous observation (69). However, RARα is not responsible for the effect of RARβ, since we did not observe any enhancement of RARα transcripts in MB231 cells that expressed introduced RARβ (data not shown). When we analyzed the expression of endogenous RARβ in cells that expressed introduced RARα, we found that it was significantly enhanced in the presence of all-*trans* RA (Fig. 6c). In our transient transfection assay, we observed that cotransfection of RARα could enhance βRARE activity in hormone-independent cells (unpublished data). Thus, the effect of RARα may be in part due to its activation of endogenous RARβ through activation of βRARE, which then triggers growth inhibition signaling. However, RARα alone may not be sufficient to render breast cancer cells responsive to RA, as was seen with BT20 cells, which express a relatively high level of RARα transcript (Fig. 2a) but which nevertheless are all-*trans* RA resistant (Fig. 2d). It is likely, therefore, that another nuclear protein(s) may participate in the regulation of RA sensitivities in breast cancer cells.

The growth-inhibitory effect of RA appears to be dependent on ER status. Whether this dependence is due to a coincidence resulting from the progression of a tumor or whether estrogen somehow influences RA activities remains to be elucidated. In our stable transfectants expressing RARβ, we did not observe



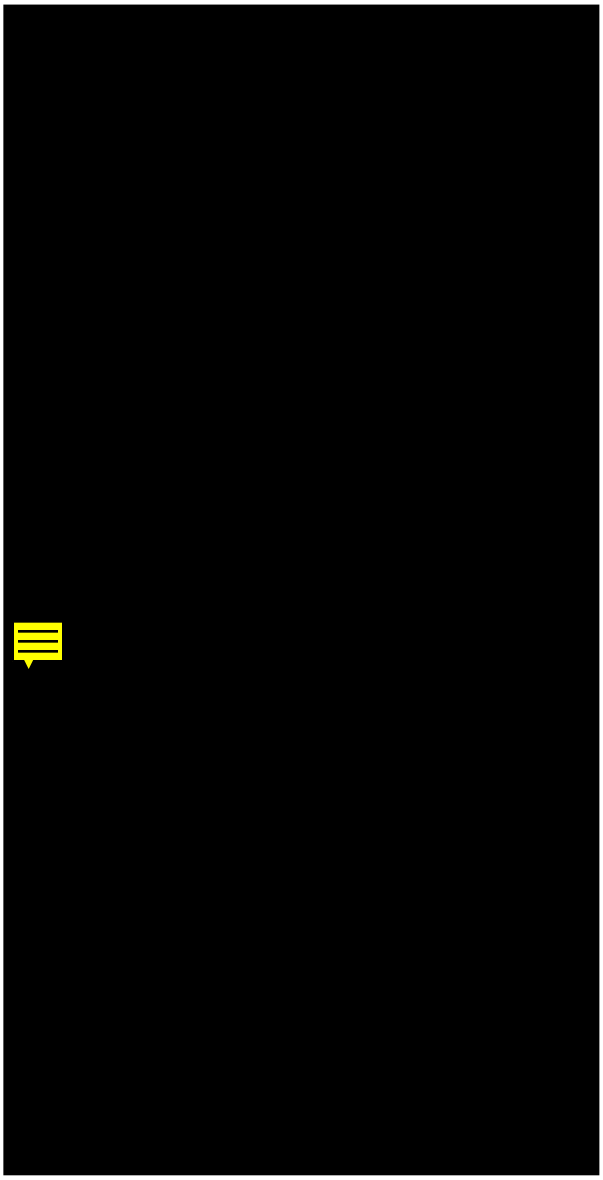
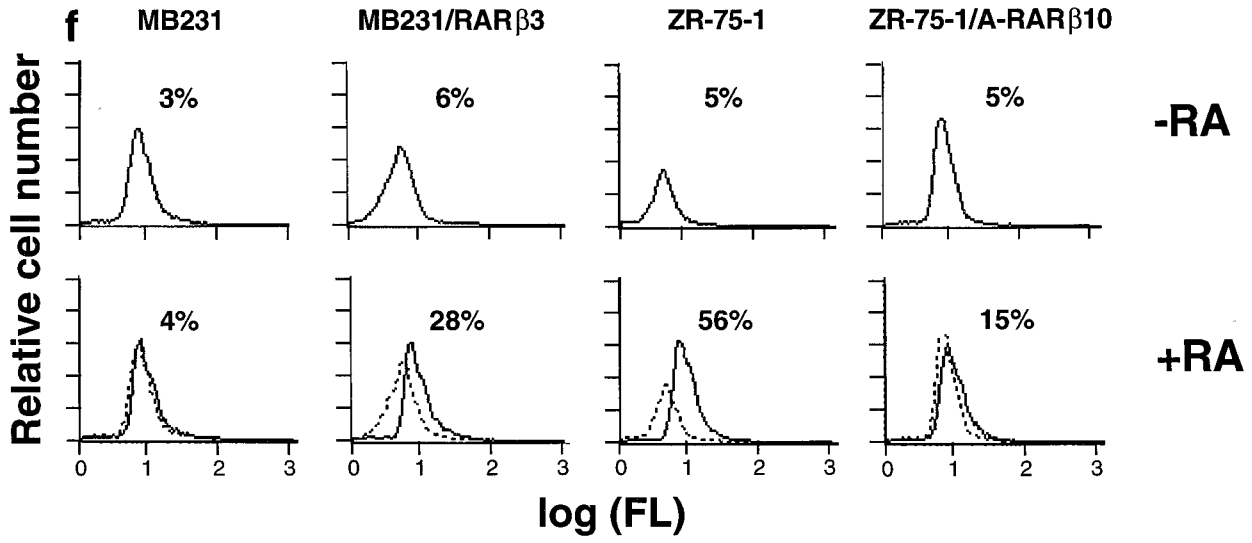
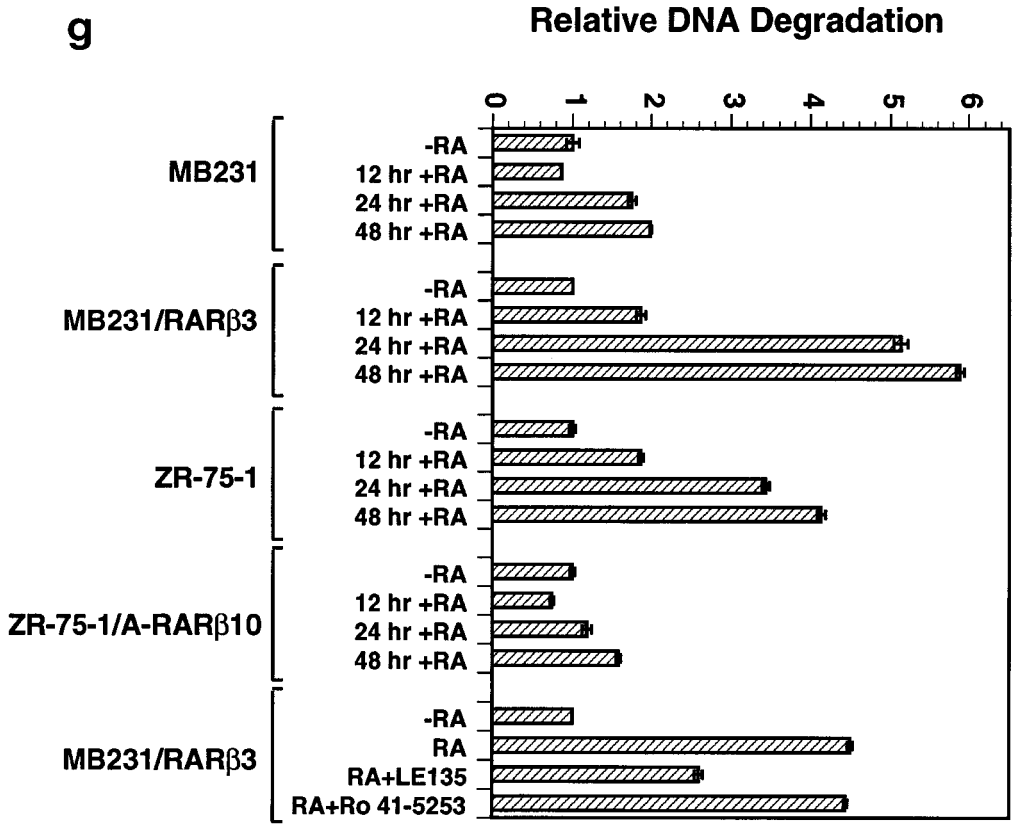


FIG. 8. RARβ promotes cell apoptosis. (a) Morphology change of MB231 cells by RARβ gene expression. (b to e) Morphological analysis of apoptotic breast cancer cells stained with propidium iodine after RA treatment. Panels b, c, d, and e represent breast cancer cell lines MB231/RARβ3, ZR-75-1, T-47D, and MCF-7, respectively. MB231/RARβ3 and ZR-75-1 cells were treated with 10^{-6} M all-*trans* RA for 48 h. T-47D and MCF-7 cells were treated with 10^{-6} M all-*trans* RA for 72 h. Arrows indicate the apoptotic nuclei. (f) TdT assays of parental and RARβ-transfected MB231 (MB231/RARβ3) cells and parental and antisense RARβ-transfected ZR-75-1 (ZR-75-1/A-RARβ10) cells after 24 h of All-*trans* RA treatment. Representative histograms show relative apoptotic cell numbers. FL, fluorescence. (g) ELISA analysis of DNA fragmentation in apoptotic breast cancer cells. Error bars show standard deviations.

any change in ER expression levels (data not shown), indicating that ER activities are not involved in RARβ-mediated growth inhibition in breast cancer cells. Recently, we have observed that RA can inhibit ER transcriptional activity in breast cancer cells (41). Rubin et al. (65) also reported that 9-*cis* RA could down-regulate ER activity in MCF-7 cells. These data demonstrate a possible cross-talk between retinoid signaling and estrogen signaling in breast cancer cells that may influence RA-induced growth inhibition in breast cancer cells.

The mechanisms by which retinoids negatively regulate growth are largely unknown. A possible mechanism is their inhibitory effect on the transcriptional regulatory activity of the proto-oncogene products cJun and cFos, the components of AP-1, which are commonly associated with cell proliferation (67, 79). In this study, we provide evidence that induction of apoptosis by RARβ may represent another important mechanism by which RA exerts its growth-inhibitory function. Apoptosis, as a distinct form of cell death, is an important process that can lead to tumor regression, and suppression of apoptosis is often associated with abnormal cell survival and malignant growth. Apoptosis of lymphoblasts induced by steroid hormones, such as glucocorticoid, has been suggested to account for the antileukemic activity of these agents (74). Retinoids were also reported to induce cell death in hematopoietic cells (58) and in breast cancer cells (61, 68). How retinoid-induced apoptosis is mediated remains to be seen. The observations (16, 54) that the expression of RARβ in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (38) imply that RARβ could be associated with programmed cell death. In this study, we observed RA-induced apoptosis in ZR-75-1 cells that highly expressed RARβ. Inhibition of RARβ activity by the expres-





sion of RARβ antisense RNA reduced the number of apoptotic cells (Fig. 8g). In contrast, RA-induced apoptosis was only observed in hormone-independent cells when RARβ was introduced and expressed in the cells (Fig. 8g). These data clearly demonstrate that RARβ is able to mediate RA-induced apoptosis of breast cancer cells.

RARβ is expressed in normal breast tissue and in normal human breast cell lines (72). In our study, all-*trans* RA-induced RARβ expression was found only in hormone-dependent breast cancer cell lines and not in independent cell lines. Since hormone-independent breast cancer cells are usually considered to represent those at a late stage of breast tumor progression, this observation suggests that the loss of RARβ gene expression may have important pathogenic consequences during the development of human breast cancer. Alteration of RARβ activities may be involved in the development of human liver cancer (12). In lung and breast cancer, a deletion of the short arm of chromosome 3p, a region that maps close to the RARβ gene, occurs with high frequency (15, 59, 80). Abnormally low levels of RARβ gene expression were observed in many human lung cancer cell lines and other cancer cell lines (22, 30, 32, 60, 86) and were suggested to contribute to the

neoplastic progression of human oral squamous cell carcinoma cell lines (32) and the tumorigenicity of human papillomavirus type 18-transformed HeLa cells (3). Furthermore, RARβ has been shown to function as a tumor suppressor gene in epidermoid lung carcinoma cells (31). These observations, therefore, suggest that a low expression level of the RARβ gene may be an important contributing factor for cancer development. The present finding that RARβ can promote apoptosis suggests that the loss of RARβ activities could remove such a negative control mechanism, resulting in uncontrolled cell proliferation and, therefore, an enhancement of the transformed phenotype of the cells.

In conclusion, our results demonstrate that the loss of RARβ gene expression and regulation by all-*trans* RA is a common feature associated with hormone-independent breast cancer cells and may be one of the major factors responsible for diminished retinoid sensitivity during the progression of a breast tumor. Thus, the expression level of the RARβ gene and its response to RA may serve as diagnostic factors for cancer and may be also used to determine whether patients with breast cancer will respond to RA treatment. Our results also demonstrate that RARβ mediates the growth-inhibitory effect of RA in part by inducing cell apoptosis, which, when lost, may contribute to cancer development. The observation that introduction of the RARβ gene into RARβ-negative cancer cells can restore RA sensitivities provides valuable directions for the development of new strategies for the treatment of human breast cancer.

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ADDENDUM

Seewaldt et al. have recently reported that RARβ can mediate growth arrest and induce apoptosis in human breast cancer cells (67a); their results support our discovery in this study.

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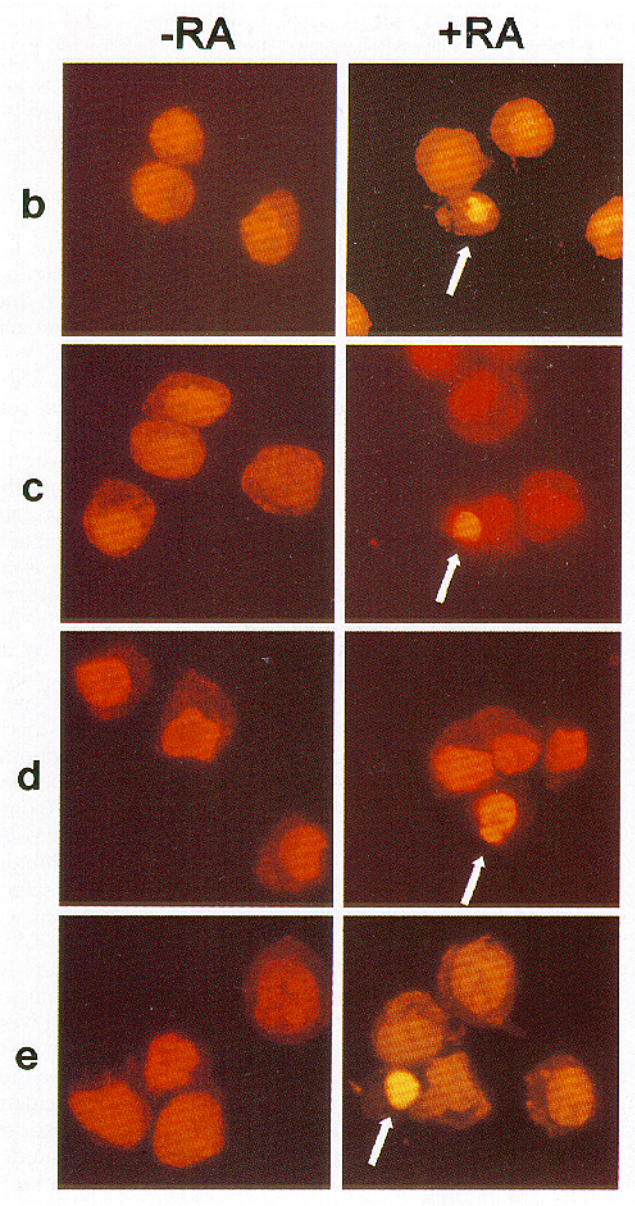
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