Synergistic Effects of Retinoic Acid and 8-Chloro-Adenosine 3′,5′-Cyclic Monophosphate on the Regulation of Retinoic Acid Receptor β and Apoptosis: Involvement of Mitochondria

Rakesh K. Srivastava,1, 2 Aparna R. Srivastava, Yoon S. Cho-Chung, and Dan L. Longo

Laboratory of Immunology, National Institute on Aging, NIH, Baltimore, Maryland 21224-6825 [R. K. S., D. L. L.], and Medicine Branch [A. R. S.] and Cellular Biochemistry Section [Y. S. C-C.], Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

In advanced or recurrent malignant diseases, retinoic acid (RA) is not effective, even at doses that are toxic to the host. In late stages of breast cancer, patients do not respond to RA because the expression of RA receptor β (RARβ) is lost. In the present study, the intracellular mechanism(s) of synergistic effects of RA and a site-selective cyclic AMP (cAMP) analogue, 8-chloro-adenosine 3′,5′-cyclic monophosphate (8-Cl-cAMP), on growth inhibition and apoptosis in breast cancer cells was examined. Our data demonstrated that hormone-dependent MCF-7 cells, but not hormone-independent MDA-MB-231 cells, are sensitive to RA-induced growth inhibition and apoptosis. Introduction of the RARβ gene into MDA-MB-231 cells resulted in a gain of RA sensitivity. 8-Cl-cAMP acted synergistically with all-trans-RA in inducing and activating RARβ gene expression that correlates with the reduction in mitochondrial membrane potential, redistribution of cytochrome c, activation of caspases, cleavage of poly(ADP-ribose) polymerase and DNA-dependent protein kinase (catalytic subunit), and induction of apoptosis. Mutations in the cAMP response element-related motif within the RARβ promoter resulted in loss of synergy in RARβ transcription. In addition, inhibition of RARβ expression by an antisense construct also blocked the antitumor effects of RA + 8-Cl-cAMP. Thus, RARβ can mediate RA and/or cAMP action in breast cancer cells by promoting apoptosis. Therefore, loss of RARβ expression may contribute to the tumorigenicity of human mammary epithelial cells. These findings suggest that RA and 8-Cl-cAMP act in a synergistic fashion and may have potential for combination biotherapy for the treatment of malignant diseases.

INTRODUCTION

Chemoprevention by agents that delay, reverse, or block cancer development is a promising approach to the cancer problem (1). Retinoids (natural and synthetic) are known to possess antiproliferative, differentiative, and immunomodulatory properties (2). A growing body of evidence from clinical research supports the concept that retinoids are useful substances in the prevention and treatment of cancer. Retinoids, either alone or in combination with biological response modifiers or chemotherapy, have proven to be effective against skin diseases including some cancers of the skin, acute promyelocytic leukemia, cervical cancer, and other malignancies (2). Furthermore, it is believed that physiological levels of retinoids guard the organism against the development of pre-malignant and malignant lesions. Retinoid therapy has been shown to prevent the development of second primary cancers among patients with head and neck cancer and lung cancer (3, 4).

Retinoids exert their modulatory effects on cell growth by binding to the retinoid receptor nuclear proteins, of which there are two classes (the RARs3 and the RXRs), each of which has three subtypes (α, β, and γ; Refs. 1 and 2). These receptors display distinct patterns of expression during development and differentiation (1, 2), suggesting that each of them may have distinct and specific functions. All-trans-RA specifically binds and activates RARs, whereas 9-cis-RA binds and activates both RARs and RXRs. In DNA binding and transcriptional activation by ligand, retinoid receptors function as heterodimers of RXR and RAR or as RXR homodimers (2, 5). The retinoid receptors may influence gene transcriptional activation by binding to specific DNA sequences (RAREs and retinoid X response elements; Ref. 6).

One of the target genes of retinoid receptors is the gene encoding RARβ (5). In its promoter region, a DR5 RARE named β-RARE was identified that mediates RA-induced RARβ gene expression in many different cell types (7). Auto-

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2 To whom requests for reprints should be addressed, Laboratory of Immunology, National Institute on Aging, NIH, Box 28, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825. Phone: (410) 558-8480; Fax: (410) 558-8284.

3 The abbreviations used are: RAR, retinoic acid receptor; RA, retinoic acid; PKA, cAMP-dependent protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; DNA-PK, DNA-dependent protein kinase; DiOC6(3), 3,3′-dihexyloxacarbocyanine iodide; 8-Cl-cAMP, 8-chloro-adenosine 3′,5′-cyclic monophosphate; TTNPB, (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthyl)-1-propenyl]benzoic acid; cAMP, cyclic AMP; CRE, cAMP response element; CREB, cAMP-responsive element binding protein; RXR, retinoid X receptor; RARE, retinoic acid response element; CAT, chloramphenicol acetyltransferase; PMSF, phenylmethylsulfonyl fluoride; DNA-PKcs, DNA-PK catalytic subunit; MPT, mitochondrial permeability transition.
regulation of the RARβ gene presumably plays a critical role in amplifying the RA response. Recently, several studies have reported the loss of RARβ expression in a range of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, and breast carcinoma (8–13). Altered retinoid receptors can result in abnormal cellular differentiation pathways and a loss of the antiproliferative effects of retinoids. This concept is supported by the observation that introduction of RARβ into RA-insensitive cell lines restored RA sensitivity in breast cancer cell lines (14). Furthermore, several studies have reported that retinoids can induce apoptosis in several different cell types (6, 15, 16). Alteration of retinoid receptor activity may therefore lead to suppression of apoptosis and result in the pathological accumulation of aberrant cells and, ultimately, in neoplasia.

The actions of cAMP are well known in the regulation of various cellular functions, including cell proliferation, differentiation, and gene induction, through the activation of PKA (17, 18). We and others (17, 19–21) have recently demonstrated that down-regulation of PKA type I by the cAMP analogue 8-Cl-cAMP and inhibition of expression of the regulatory subunit of PKA type Iα by an antisense construct induce apoptosis in several cancer cell lines. Furthermore, intracytoplasmic microinjection of purified PKA catalytic subunit commits the cells to death (22). The CRE-related motif, TGATGTCA at position −99 to −92, is able to enhance RA-dependent RARβ promoter activation (23). Based on these data, it is likely that RARβ may mediate some of the effects of cAMP and RA in regulating cellular responses.

For advanced or recurrent malignant diseases, RA is not very effective, even at doses that are toxic to the host. Thus, the development of new chemotherapeutic agents and new combination regimens is highly desirable. Synergistic effects in growth inhibition of cancer cells in culture have been observed when retinoids are administered in combination with other agents such as antiestrogen (24, 25) or IFN (26). We have reported that retinoids can induce apoptosis in several different cell types (14). Furthermore, several studies have investigated. The purpose of our studies was to examine the actions of cAMP and inhibition of expression of the regulatory subunit of PKA type Iα by an antisense construct induce apoptosis in several cancer cell lines. Furthermore, intracytoplasmic microinjection of purified PKA catalytic subunit commits the cells to death (22). The CRE-related motif, TGATGTCA at position −99 to −92, is able to enhance RA-dependent RARβ promoter activation (23). Based on these data, it is likely that RARβ may mediate some of the effects of cAMP and RA in regulating cellular responses.

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against 100 volumes of buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, and 2 mM DTT] for 4 h. The extracts were then centrifuged, and the final protein concentration was determined by the Bio-Rad protein assay kit. The oligonucleotide probe for β-RARE (GGG-TAGGGTTCACCGAAAAGTTCACTCG) was synthesized commercially. The DNA-protein binding assay was conducted in a volume of 20 μl. In brief, 10 μg of protein were incubated with 1 μg of polydeoxyinosinic dCMP and 0.5–1.0 ng of [32P]-labeled synthetic oligonucleotide (about 1 × 10⁵ cpm) in a binding buffer [10 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, and 1 mM DTT] for 20 min at room temperature. DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5× Tris-borate EDTA. The gels were then dried and autoradiographed with intensifying screens at −70°C.

**Transient Transfection and CAT Assay.** To measure the transcriptional activation of β-RARE in breast cancer cells, β-RARE linked with the CAT gene (β-RARE-tk-CAT) was used as a reporter gene. In brief, pβ-RARE-tk-CAT is a synthesized human β-RARE (AGGGTTCACCGAAAAGTTCACTCG) inserted into the HindIII and BamHI sites of pβLCAT2. This latter plasmid was used as a negative control in transient transfection with RA to show that induction did not derive from the vector. β-RARE-tk-CAT (2.0 μg) and 3.0 μg of β-galactosidase expression vector (pCH 110; Pharmacia) were transiently transfected into cells along with LipofectAMINE (Life Technologies, Inc.). Cells were grown in the presence or absence of all-trans-RA. Transfection efficiency was normalized by β-galactosidase activity.

**Stable Transfection.** cDNA for the RARβ gene was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, CA). The RARβ antisense expression vector was provided by Dr. X-K. Zhang (La Jolla Cancer Research Foundation, La Jolla, CA). In brief, to construct the RARβ antisense expression vector, cDNA for the RARβ gene was cloned into the pRc/CMV expression vector in an antisense orientation (14). The resulting recombinant constructs were then stably transfected into breast cancer cells along with LipofectAMINE and selected with 400 μg of G418 (Life Technologies, Inc.). The expression of exogenous RARβ gene was determined by Northern blot analyses (data not shown).

**Measurement of Mitochondrial Energization.** Mitochondrial energization was determined as the retention of the dye DiOC₆(3). Cells (5 × 10⁵) in 500 μl of complete RPMI 1640 were loaded with 100 nM DiOC₆(3) during the last 30 min of treatment. The cells were then pelleted at 700 × g for 10 min. The supernatant was removed, and the pellet was resuspended and washed in PBS two times. The pellet was then lysed by the addition of 600 μl of deionized water, followed by homogenization. The concentration of retained DiOC₆(3) was determined on a fluorescence spectrometer (Cyto Fluor PerSeptive Biosystems, Framingham, MA) at 488 nm excitation and 510 nm emission (28).

**Subcellular Fractionation.** Mitochondrial and cytosolic (S100) fractions were prepared by resuspending cells in 0.8 ml of ice-cold buffer [250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml PMSF, 8 μg/ml aprotinin, and 2 μg/ml leupeptin (pH 7.4)] (29). Cells were passed through an ice-cold cylinder cell homogenizer. Nuclei were pelleted by a 10-min, 750 × g spin. The supernatant was spun at 10,000 × g for 25 min. This pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was spun at 100,000 × g for 1 h. The supernatant from this final centrifugation represents the S100 (cytosolic) fraction.

**DNA-PK Activity Assay.** DNA-PK assays were performed according to manufacturer’s instructions (Promega). In brief, assays were performed in a final volume of 20 μl containing 25 mM HEPES-KOH (pH 7.5), 4 mM MgCl₂, 13 mM spermidine, 1 mM DTT, 5% glycerol, 0.1% NP40, 2 mM ATP, 50 mM KCl, and [32P]ATP (3000 Ci/mmol; 0.5 μl). The substrate for DNA-PK phosphorylation was a synthetic peptide (EPPLSQEAFADLWKK) and was present in reactions at a concentration of 500 μM. Each sample was also assayed in the presence of the nonsubstrate peptide EPPLSEQFADLWKK. The reaction mixture was incubated for 10 min at 30°C, and the reaction was stopped by adding 20 μl of 30% acetic acid. Ten μl of the reaction products were spotted onto a 2 × 2-cm piece of Whatman P-81 phosphocellulose paper. Filters were air-dried, washed four times with 15% acetic acid, and counted in a scintillation counter.

**RESULTS**

**Synergistic Inhibitory Effects of RA and 8-Cl-cAMP on Cell Viability.** To establish the involvement of RAR and RXR in RA-induced growth inhibition in hormone-dependent MCF-7 cells, we used retinoids selective for RXR homodimers and RAR-RAR heterodimers. TTNPB (30) and (all-E)-UAB30 (31), which specifically bind RARs and activate RXR-RAR heterodimers, inhibited cell viability at a level similar to that observed with all-trans-RA, whereas (9z)-UAB8 and (9z)-UAB30 (31), which specifically activate RXR homodimers, had no effect on cell viability (Fig. 1A). These data, along with others (14), suggest that ligands that bind to RARs are mainly...
Fig. 1  Cell viability of hormone-independent MDA-MB-231 and hormone-dependent MCF-7 cells. A, activation of RARs but not RXRs is required for RA-induced growth inhibition in MCF-7 cells. The effects of RAR-RXR heterodimer [(all-E)-UAB30 and TTNPB] and RXR homodimer [(9z)-UAB8 and (9z)-UAB30]-specific activators on the growth of MCF-7 cells are shown in graphic form. The effect of all-trans-RA is shown for the purpose of comparison. Cells were seeded at 1000 cells/well and treated with 100 nM retinoids for 5 days. B, dose-dependent inhibition of cell viability by 8-Cl-cAMP. Cells were treated with 8-Cl-cAMP (1–15 μM) for 5 days. C, effects of RA (10^{-10} to 10^{-4} M) on cell viability in MDA-MB-231 cells. Cells were treated with 9-cis-RA, 13-cis-RA, or all-trans-RA for 5 days. D, dose-dependent inhibition of cell viability by RA (10^{-10} to 10^{-4} M) in MCF-7 cells. Cells were treated with 9-cis-RA, 13-cis-RA, or all-trans-RA for 5 days. E and F, synergistic effect of all-trans-RA and 8-Cl-cAMP on growth inhibition. Cells were treated with 8-Cl-cAMP (1 μM) and all-trans-RA (1 nM) singly and in the presence of various concentrations of the other drug at day 0, and the cell number was counted at day 5. The data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. The percentage growth inhibition values for 1 μM 8-Cl-cAMP (E) and 1 nM all-trans-RA (F) when added alone. The height of the bars on the left of each pair represents the sum of the individual drug effects or the expected percentage of growth inhibition if analogues were added together. The total heights of the solid bars indicate the observed percentage of growth inhibition when drugs were added in combination at the indicated concentrations. The differences between the heights of the paired bars reflect the magnitude of synergism of growth inhibition. Data (mean ± SE) represent one of three separate experiments that gave similar results. The insets show the synergism quotient at each concentration of drug combination. The synergism quotient was defined as the net growth-inhibitory effect of a drug combination divided by the sum of the net individual drug effects on growth inhibition.
responsible for the RA-induced growth inhibition of MCF-7 cells.

To evaluate the responsiveness of hormone-dependent (MCF-7) or hormone-independent (MDA-MB-231) breast cancer cells to either RA (9-cis-RA, 13-cis-RA, or all-trans-RA) or cAMP analogue 8-Cl-cAMP, we analyzed the viability of treated cells by MTT assay. Treatment of both MDA-MB-231 and MCF-7 cells with 8-Cl-cAMP resulted in inhibition of cell viability in a dose-dependent manner (Fig. 1B). Treatment of hormone-independent MDA-MB-231 cells with RA (9-cis-RA, 13-cis-RA, or all-trans-RA) had no effect on cell viability (Fig. 1C). By comparison, treatment of hormone-dependent MCF-7 cells with RA (9-cis-RA, 13-cis-RA, or all-trans-RA) resulted in reduction of cell viability in a dose-dependent manner (Fig. 1D). The 9-cis-RA was modestly more effective than either 13-cis-RA or all-trans-RA in inhibiting the cell viability (Fig. 1D). Because RARβ contains a CRE-related motif (TGATGTCA) in its promoter region, we next evaluated the interactive effects of low doses of 8-Cl-cAMP and all-trans-RA on cell viability. The synergism quotients of interactive effects of 8-Cl-cAMP and all-trans-RA were determined by keeping the concentration of one drug constant and varying the concentration of other drug, and vice versa (Fig. 1, E and F). When given separately, treatment of MCF-7 cells with either all-trans-RA (1 nM) or 8-Cl-cAMP (1 μM) had slight effects on the cell viability (Fig. 1, E and F). The combination of 1 μM 8-Cl-cAMP and 1 nM all-trans-RA yielded the largest synergistic effect.

Cleavage of PARP and DNA-PKcs in RA- and 8-Cl-cAMP-treated Cells. Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation (32). Recently, PARP cleavage has been used as an index of apoptosis induced by a variety of anticancer drugs (33, 34). Therefore, we measured the cleavage of PARP to confirm that cells were undergoing apoptosis upon drug treatment. Combined treatment with RA (9-cis-RA, 13-cis-RA, or all-trans-RA, 1 nM) + 8-Cl-cAMP (1 μM) resulted in cleavage of p116PARP to its M₆,85,000 fragment in MDA-MB-231 cells (Fig. 2A). By comparison, cleavage of PARP was not observed with low doses of 8-Cl-cAMP or RA or in untreated control cells (Fig. 2A).

DNA-PK, which is involved in double-stranded DNA break repair, might be expected to be preferentially degraded during the DNA fragmentation that accompanies apoptosis (35). To determine whether DNA-PKcs might be a target for proteolytic degradation during DNA damage-induced apoptosis (35), we used immunoblotting with antibody targeted against the catalytic subunit with extracts from MDA-MB-231 cells treated with RA and/or 8-Cl-cAMP. The treatment of MDA-MB-231 cells with a low dose of either RA (9-cis-RA, 13-cis-RA, or all-trans-RA; 1 nM) or 8-Cl-cAMP (1 μM), followed by immunoblotting, had no effect on DNA-PKcs degradation (Fig. 2B). However, combined treatment of MDA-MB-231 cells with RA + 8-Cl-cAMP resulted in DNA-PKcs degradation to the M₆,240,000 fragment (Fig. 2B). Because DNA-PKcs must associate with Ku, the DNA-targeting component of the complex, to be activated (36), we also determined the fate of Ku-70 and Ku-86 in MDA-MB-231 cells during apoptosis. There was no evidence for the degradation of either protein in MDA-MB-231 cells exposed to RA and/or 8-Cl-cAMP (Fig. 2C).

To determine whether the cleavage of DNA-PKcs interferes with its function, we measured the activity of DNA-PK in MDA-MB-231 cells treated with all-trans-RA and 8-Cl-cAMP using a peptide substrate (37). It is evident from the results in Fig. 2D that kinase activity is reduced to less than 25% upon treating cells with a combination of all-trans-RA + 8-Cl-cAMP in MDA-MB-231 cells. The loss of DNA-PK activity therefore
correlates with the degradation of DNA-PKcs. These results confirmed our findings that RA and 8-Cl-cAMP act in a synergistic fashion to induce apoptosis in breast cancer cells.

**Redistribution of Cytochrome c in RA + 8-Cl-cAMP-treated Cells.** Mitochondrial dysfunction has been observed in the early stages of apoptosis (3). Both mitochondrial depolarization and the loss of cytochrome c from the mitochondrial intermembrane space to the cytosol have been proposed as early central events in apoptotic cell death (38–40). Recently, cytochrome c release has been shown to activate caspases, thus continuing transmission of the apoptotic program (39). We therefore determined the presence of cytochrome c in the cytosolic and mitochondrial fractions of cells treated with all-trans-RA and/or 8-Cl-cAMP (Fig. 3A). Treatment of MDA-MB-231 cells with a low dose of either all-trans-RA (1 nM) or 8-Cl-cAMP (1 μM) had no effect on cytochrome c release from the mitochondria to the cytosol (Fig. 3A). Combined treatment of all-trans-RA + 8-Cl-cAMP caused a redistribution of cytochrome c from the mitochondria to the cytosol in MDA-MB-231 cells (Fig. 3A). Similarly, cytochrome c release from the mitochondria to the cytosol was observed in MDA-MB-231 cells due to combined treatment of all-trans-RA + 8-Cl-cAMP.

MPT refers to the regulated opening of a large, nonspecific pore in the inner mitochondrial membrane (38). The MPT causes the loss of the mitochondrial membrane potential (ΔΨm) in cells undergoing apoptosis (41). The fluorescent dye DiOC6(3) localizes to the mitochondria, and the MPT reduces the accumulation of DiOC6(3) as a consequence of the loss of ΔΨm (42). Treatment of MDA-MB-231 cells with all-trans-RA + 8-Cl-cAMP produced a steady decline in ΔΨm (Fig. 3B), which began after 10 h. By comparison, treatment of MDA-MB-231 cells with either all-trans-RA or 8-Cl-cAMP had no significant effect (Fig. 3B). These data suggest that loss of mitochondrial membrane potential (Fig. 3B) precedes caspase activation and that the caspases act downstream of mitochondrial apoptotic events.

**Induction of RARβ by Combined Treatment with RA and 8-Cl-cAMP.** To determine which RAR subtype is involved in RA-induced growth inhibition, we investigated the expression of RAR (α, β, and γ) and RXR (α, β, and γ) in MDA-MB-231 and MCF-7 cells. Both cell types expressed RARα, RARγ, RXRα, RXRβ, and RXRγ, but not RARβ, under the conditions used (Fig. 4A). Because these cells did not express detectable levels of RARβ, we examined whether the expression of RARβ could be induced by all-trans-RA in MCF-7 and MDA-MB-231 breast cancer cells. In MCF-7 cells, all-trans-RA (1 μM) was able to induce RARβ mRNA, whereas in MDA-MB-231 cells, it was ineffective (Fig. 4B). Because combined treatment of MDA-MB-231 cells with all-trans-RA and 8-Cl-cAMP inhibited cell viability and induced apoptosis in a synergistic fashion, it was of interest to examine whether this synergistic effect was due to increased expression of the RARβ gene because its promoter contains a functionally active CRE-related motif (23). Treatment of MDA-MB-231 and MCF-7 cells with a low dose of all-trans-RA (1 nM) had no effect on the induction of RARβ mRNA (Fig. 4C). By comparison, a low dose of 8-Cl-cAMP (1 μM) led to a modest elevation in RARβ mRNA. Interestingly, combined treatment with these low doses of 8-Cl-cAMP and all-trans-RA had a synergistic effect on the induction of RARβ mRNA in both MDA-MB-231 and MCF-7 cells (Fig. 4C). These data suggest that the induction of RARβ may be responsible for the synergistic effects of all-trans-RA and 8-Cl-cAMP.

**Abnormal Transcriptional Regulation of β-RARE in Hormone-independent MDA-MB-231 Cells.** It is known that all-trans-RA-induced RARβ expression is mediated by the β-RARE present in the RARβ promoter (7, 43, 44). The failure of RA to induce RARβ gene expression in a hormone-independent MDA-MB-231 breast cancer cell line suggests that the regulation of RARβ expression by RA is disrupted. Because the RARβ promoter contains a CRE-related motif, and RARβ expression was enhanced with combined treatment of all-trans-RA and 8-Cl-cAMP, it was of interest to examine the transcriptional regulation of the β-RARE by RA and/or 8-Cl-cAMP. A CAT reporter construct containing β-RARE linked with a thymidine kinase promoter (β-RARE-tk-CAT) was used as a reporter construct to determine the degree of all-trans-RA response with or without 8-Cl-cAMP in the hormone-independent MDA-MB-231 cells by transient transfection assays (Fig. 5). When this
reporter was transfected into MDA-MB-231 cells, no CAT activity in response to all-trans-RA was observed (Fig. 5A). In contrast, only a slight but significant induction of CAT activity was seen in MDA-MB-231 cells in response to 8-Cl-cAMP. Interestingly, a strong induction of CAT activity was observed in response to combined treatment with all-trans-RA and 8-Cl-cAMP (Fig. 5A). These results suggest that the loss of RARβ expression in MDA-MB-231 cells may be due to an abnormal transcriptional regulation of β-RARE.

To investigate whether the loss of the β-RARE activity is due to altered β-RARE binding, MDA-MB-231 cells were treated with all-trans-RA with or without 8-Cl-cAMP and analyzed by gel shift for their binding to β-RARE. One binding complex was formed in untreated MDA-MB-231 cells and after treatment with all-trans-RA (1 nM) and/or 8-Cl-cAMP (1 μm) for 5 days, and binding complexes were analyzed by gel-shift assays. In Lanes 5 and 6, nuclear extracts were incubated in the presence of an anti-CREB antibody. The arrows indicate the specific binding complex present in breast cancer cells.
(Fig. 5B), treatment of MCF-7 cells with 8-Cl-cAMP resulted in a darker band (Fig. 5C, Lane 2) compared to untreated control cells (Fig. 5C, Lane 1). The combined treatment of MCF-7 cells with all-trans-RA + 8-Cl-cAMP caused the appearance of an additional band (Fig. 5C, Lane 4). The addition of anti-CRE antibody to the nuclear extract of 8-Cl-cAMP-treated cells caused a supershift (Fig. 5C, Lane 6). These data suggest that CRE acts in a cooperative manner with β-RARE in MCF-7 cells.

**Recovery of RA Sensitivity in Hormone-independent Breast Cancer Cells by RARβ Expression.** Induction of RARβ by all-trans-RA may be responsible for the RA-induced growth inhibition in hormone-dependent MCF-7 breast cancer cells, and the loss of RA sensitivity in hormone-independent breast cancer cells may be due to a lack of RARβ or low levels of RARβ in MDA-MB-231 cells. To test this hypothesis, MDA-MB-231 cells were stably transfected with either vector alone (MDA-MB-231/vector) or vector containing cDNA for the RARβ gene (MDA-MB-231/RARβ). To determine the effect of the exogenous RARβ gene, the viability of cells transfected with MDA-MB-231/RARβ and cells transfected with empty vector (MDA-MB-231/vector) was measured in the presence or absence of RA (9-cis-RA, 13-cis-RA, or all-trans-RA) by MTT assay (Fig. 6, A and B). MDA-MB-231 cells transfected with empty vector (MDA-MB-231/vector) did not show any response to RA (9-cis-RA, 13-cis-RA, and all-trans-RA; Fig. 6A). In contrast, RA (9-cis-RA, 13-cis-RA, or all-trans-RA) inhibited viability in MDA-MB-231/RARβ-transfected cells (Fig. 6B). These data suggest that the growth-inhibitory effect of RA is mediated by the RARβ product and that expression of RARβ can restore RA sensitivity in hormone-independent breast cancer cells.

To further characterize the effect of the transfected RARβ gene, RARβ-transfected cells were analyzed for the induction of the apoptosis by RA and/or 8-Cl-cAMP. All-trans-RA induced apoptosis in a dose-dependent manner in transfected cells that expressed the RARβ (MDA-MB-231/RARβ) gene (Fig. 6C); MDA-MB-231/vector-transfected cells were not responsive to RA treatment (data not shown). Because combined treatment of MDA-MB-231 cells with all-trans-RA and 8-Cl-cAMP resulted in induction of RARβ and apoptosis, we evaluated the contribution of RARβ receptors in the process of apoptosis by transfecting the cells with RARβ antisense (Fig. 6D). Combined treatment of MDA-MB-231 cells with all-trans-RA and 8-Cl-cAMP induced apoptosis in controls and in cells transfected with sense RARβ (Fig. 6D). As expected, antisense RARβ inhibited the effects of combined treatment of all-trans-RA and 8-Cl-cAMP on apoptosis in MDA-MB-231 cells (Fig. 6D). Because exogenous expression of RARβ restores RA sensitivity in MDA-MB-231 cells, we next examined the specificity of RARβ actions by transfecting cells with antisense RARβ (Fig. 6E). As expected, antisense-RARβ abrogated all-trans-RA effects on apoptosis in MDA-MB-231/RARβ cells (Fig. 6E). Similarly, antisense RARβ was able to inhibit all-trans-RA-induced apoptosis in MCF-7 cells (Fig. 6F). Together, these data demonstrate that all-trans-RA can induce apoptosis in breast cancer cells in the presence of RARβ, and these effects of all-trans-RA on apoptosis can be enhanced by 8-Cl-cAMP. Furthermore, RARβ expression is critical to these effects; sensitive cells became resistant in the absence of RARβ.

**Recovery of Normal Transcriptional Regulation of β-RARE in Hormone-independent MDA-MB-231 Cells Overexpressing Exogenous RARβ.** All-trans-RA-induced RARβ expression is mediated by the β-RARE present in the RARβ promoter (7, 43, 44). The loss of the RA effect in inducing RARβ gene expression in the hormone-independent human breast cancer cell line suggests that the regulation of RARβ expression by RA is disrupted. Because transfection of the RARβ gene in hormone-independent MDA-MB-231 cells resulted in a gain of apoptotic function of all-trans-RA (Fig. 6), it was of interest to determine whether these cells also possessed normal RA-induced transcriptional activity. To further examine the impaired RA response, a CAT reporter construct containing β-RARE linked with a thymidine kinase promoter (β-RARE-tk-CAT) was used as a reporter construct to determine the degrees of RA response in both hormone-dependent and -independent breast cancer cells expressing the exogenous RARβ gene (Fig. 7). All-trans-RA treatment alone had no significant increase in CAT activity over untreated control in MDA/vector cells, whereas it caused a significant increase in CAT activity in MDA/RARβ cells (Fig. 7A). Furthermore, although somewhat effective alone, 8-Cl-cAMP acted synergistically with all-trans-RA in inducing CAT activity. Similarly, synergistic interactions between all-trans-RA and 8-Cl-cAMP in inducing CAT activity were noticed in MCF-7/Vect and MCF-7/RARβ cells (Fig. 7A). These data suggest that exogenous RARβ was transcriptionally functional in mediating all-trans-RA and 8-Cl-cAMP effects in breast cancer cells.

The presence of a putative cis-acting element, the CRE-related sequence at position −99 to −92 in human RARβ2 promoter, has been reported previously (23). This sequence (TGAGTGCTA) differs from the eight-base consensus CRE sequence TGGCGCTA (45) by one base substitution (C→T) at position four. In addition, this element is functionally active as a CRE because it can bind members of the CREB/activating transcription factor transcription factor family and mediates the stimulatory effect of cAMP on RA-dependent RARβ2 promoter activation in human fetal kidney 293 cells (46). Because we observed synergistic effects between 8-Cl-cAMP and RA on RARβ activation, it was of interest to determine the functional contribution of CRE in RARβ2 activation. To answer this question, a −180/+156 CAT reporter construct was generated in which the CRE motif was disrupted by site-directed mutagenesis (Fig. 7B), and the responses to RA and/or cAMP were determined in MDA-MB-231 cells transfected with the RARβ gene. As can be seen in Fig. 7C, treatment of cells with 8-Cl-cAMP or forskolin resulted in the induction of CAT activity. Interestingly, 8-Cl-cAMP or forskolin acted synergistically with all-trans-RA in trans-activating RARβ2. Furthermore, mutation of the CRE completely abolished the synergistic effects of 8-Cl-cAMP/forskolin + all-trans-RA on RARβ2 transactivation (Fig. 7C). These results demonstrate that the CRE is responsible for the enhancement of RA-dependent RARβ2 promoter activation that we observed on the −180/+156 promoter region.
DISCUSSION

In the present study, we have demonstrated that the combined treatment of human breast cancer cells with RA and a site-selective cAMP analogue, 8-Cl-cAMP, synergizes to kill the cells through induction of the RARβ gene. Furthermore, hormone-dependent MCF-7 cells were responsive to RA treatment alone, whereas hormone-independent MDA-MB-231 cells were insensitive to RA treatment. In addition, sensitivity to the growth-inhibitory actions of RA can be restored in the hormone-independent MDA-MB-231 cells by the induction of RARβ expression. The combination of RA and 8-Cl-cAMP resulted in a synergistic induction of the RARβ gene that was correlated with inhibition in cell viability, loss of mitochondrial potential, cytochrome c redistribution, activation of caspases, and cleavage of PARP and DNA-PKcs, and apoptosis. This is the first report showing that RA and the cAMP analogue 8-Cl-cAMP act synergistically in suppressing cancer cell growth and inducing apoptosis through regulation of the RARβ gene.

Many of the effects of retinoids result from modulation of gene expression. Nuclear retinoid receptors, ligand-activated transcription enhancing factors, play a major role in mediating the effects of retinoids on gene expression and, consequently, on the growth and differentiation of both normal and tumor cells (3, 47). Changes in the expression of specific receptors could abrogate the retinoid signaling pathway and result in enhanced carcinogenesis. The role of RARβ in mediating RA-induced growth inhibition is further demonstrated by our stable transfection studies. Hormone-independent MDA-MB-231 cells that are devoid of RARβ did not show a growth-inhibitory response to RA (9-cis-RA, 13-cis-RA, or all-trans-RA). However, RARβ gene transfection into MDA-MB-231 cells resulted in a gain of responsiveness to RA (9-cis-RA, 13-cis-RA, or all-trans-RA). Thus, activation of the RARβ pathway may be critical for growth inhibition and the induction of apoptosis. Although ineffective alone, all-trans-RA acted synergistically with 8-Cl-cAMP in inducing apoptosis and trans-activating β-RARE in...
RARβ-overexpressing MDA-MB-231 cells. In our study, 8-Cl-cAMP synergizes the effects of RA by enhancing the expression of RARβ, possibly because the RARβ promoter contains a functional CRE-related motif (TGATGTCA; Ref. 23). Together, these data clearly demonstrate that RARβ can mediate the all-trans-RA-induced growth inhibition and apoptosis in breast cancer cells. In support of this hypothesis, a recent study has demonstrated that RARβ expression increases in normal breast cells (undergoing replicative senescence), but not in established tumor cell lines (10).

The mechanism by which retinoids inhibit the growth of hormone-dependent but not hormone-independent mammary carcinoma cells is not well understood. As a consequence of data presented here, it can be stated that RARβ is clearly involved in this mechanism. The RARβ gene promoter includes a RARE that can be activated by RXR/RAR heterodimers. In hormone-independent breast cancer cell lines, RA does not induce RARβ gene expression, but tumor cells can trans-activate an exogenous β-RARE (10). Recently, Widschwendter et al. (48) were not able to detect any mutations within the β-RARE promoter in breast cancer cells not expressing RARβ.

It is possible that negative regulation of RARβ in tumor cells is due to a down-regulation of transcription directed by other regions of the promoter in addition to the RARE, implicating...
trans-elements. Hormones such as retinoids, glucocorticoids, and estrogens have been shown to inhibit proliferation through inhibition of the activator protein 1 (AP-1) transcription factor (49, 50). The antagonism between AP-1 and these nuclear hormone receptors may be mediated by competing or squelching essential coactivators such as CREB binding protein (46).

However, the exact mechanism of this transcription factor cross-talk is not fully understood, and more indirect mechanisms may also be involved. The communication between the upstream elements and the β-RARE seems to be disturbed in HeLa cells (51). Loss of transcription factors that induce RARβ transcription could be another reason for the complete loss of this receptor in the tumor. Ectopic expression of RARα cDNA also leads to a higher level of RARβ gene expression (14, 52).

It has been reported that a CRE-related element in the RARβ2 promoter contributes to the trans-activation of the promoter by RA (23), which is known to be mediated by a RARE located in the proximity of the TATA box (43). This CRE-like element is located at position −99 to −92 in the human RARβ2 promoter and consists of the β motif TGATGTCA, which differs by only one base (underline) from the consensus CRE, TGACGTCA. In transient transfection experiments, Kruyt et al. (23) demonstrated that forskolin was able to enhance RA-dependent RARβ2 promoter activation via a CRE-related element in human fetal kidney 293 cells, a finding that suggests that this element can mediate transactivation upon activation of the cAMP signal transduction pathway. In the present study, 8-Cl-cAMP or forskolin acted synergistically with all-trans-RA in activating RARβ, and these synergistic effects were eliminated when mutated CRE (nonfunctional) was used in the RARβ2 promoter-tk-CAT construct (Fig. 7), suggesting that these synergistic effects on RARγ gene expression were due to the CRE-related motif present in the RARβ promoter. Besides conservation of the CRE-related motif in the RARβ2 promoter, this element is also conserved in the RARα2 promoter (53).

The involvement of retinoid receptors in the regulation of gene expression and the growth suppression of breast cancer cells has been demonstrated (2, 14). In vitro experiments have demonstrated that RARβ mediates retinoid action in breast cancer cells by promoting apoptosis, and loss of RARβ may contribute to the tumorigenicity of human mammary epithelial cells (11, 14, 54). Recently, based on in vitro and in vivo experiments, RARβ has been implicated as a tumor suppressor (8, 11, 55, 56). Interestingly, transfection of a human epithelial lung cancer in vitro with a RARβ expression vector resulted in decreased tumorigenicity (8). In addition, transgenic mice expressing antisense RARβ2 developed carcinomas 14–18 months after birth (55). Abnormalities in the expression of RARβ in malignant head and neck tissues and in non-small cell lung cancer have been demonstrated previously (11, 56). In this study, we have provided convincing evidence that the induction of apoptosis by RARβ may represent another important mechanism by which RA exerts its growth-inhibitory function.

The family of caspases mediates a highly specific proteolytic cleavage early in the process of apoptosis (29, 35). Mitochondrial dysfunction, such as a reduction in ΔΨm, has also been observed in the early stages of apoptosis (40). Cytochrome c has been shown to bind apoptotic protease-activating factor-1 and, in the presence of dATP, promotes the activity of caspase-9, which in turn activates caspase-3, thus continuing transmission of the apoptotic program (39, 40). Our findings show that combined treatment of all-trans-RA + 8-Cl-cAMP resulted in the release of cytochrome c from the mitochondria to the cytosol, a reduction in ΔΨm, and the activation of caspases.

Several proteins that are cleared during apoptosis have been shown to be specific targets for caspases including DNA-PK (57) and PARP (58). DNA-PK is composed of a 460,000 catalytic subunit tentatively termed DNA-PKcs (59) and a DNA-binding component Ku protein (p70/p86; Ref. 36). It has been revealed that DNA-PK is involved at least in DNA double-strand break repair. Another enzyme implicated in DNA repair, PARP, is cleaved and inactivated during apoptosis, suggesting that some DNA repair proteins may be selectively targeted for destruction during apoptosis. In the present study, we have demonstrated that DNA-PKcs and PARP are preferentially degraded due to combined treatment with RA + 8-Cl-cAMP in cancer cells.

Because RARβ expression is absent or decreased in human breast cancer cell lines and primary tumors, the induction of apoptosis through the induction and transactivation of RARβ expression by a combination of signal transduction-modulating agents is a possible approach for cancer treatment. Induction of RARβ expression by RA + 8-Cl-cAMP was associated with the induction of apoptosis. Thus, combined treatment with RA + 8-Cl-cAMP may have therapeutic potential for epithelial malignancies in humans. Each agent has been used in humans, and combination therapy is feasible.

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Synergistic Effects of Retinoic Acid and 8-Chloro-Adenosine 3′,5′-Cyclic Monophosphate on the Regulation of Retinoic Acid Receptor β and Apoptosis: Involvement of Mitochondria


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