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RETINOIC ACID RECEPTORS AND CANCERS

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■ **Abstract** Studies utilizing experimental animals, epidemiological approaches, cellular models, and clinical trials all provide evidence that retinoic acid and some of its synthetic derivatives (retinoids) are useful pharmacological agents in cancer therapy and prevention. In this chapter, we first review the current knowledge of retinoic acid receptors (RARs) and their role in mediating the actions of retinoic acid. We then focus on a discussion of RARα and acute promyelocytic leukemia followed by a discussion of the role of RARs, in particular RARβ expression, in other cancer types. Loss of normal RAR function in the presence of physiological levels of RA (either due to alterations in the protein structure or level of expression) is associated with a variety of different cancers. In some cases treatment with pharmacological doses of RA can be effective.

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INTRODUCTION

Retinoic acid (RA), the most potent natural form of vitamin A, plays an important role in mediating the growth and differentiation of both normal and transformed cells (for review, see 32). It is essential for many diverse biological functions

including growth, vision, reproduction, embryonic development, differentiation of epithelial tissues, and immune responses. In addition, data from a variety of different areas of investigation including experimental animals, epidemiology, cellular models, and clinical trials all provide evidence supporting the use of RA and synthetic derivatives (termed retinoids) as pharmacological agents in cancer therapy and prevention (for review, see 44, 74, 76). Because the regulation of growth and differentiation of normal, premalignant, and malignant cells is generally thought to result from direct and indirect effects on gene expression, and RA has been shown to function by transcriptionally regulating gene expression via retinoic acid receptors (RARs) and retinoid X receptors (RXRs), there has been great interest in examining the role of RARs and RXRs in the development of cancer and in the treatment/prevention of cancer.

In this chapter we first briefly review the current knowledge concerning RARs and the mechanism of transcriptional regulation by these receptors. We then focus on RARα and acute promyelocytic leukemia (APL). Translocations involving the RARα gene are a hallmark of APL. These translocations result in the expression of fusion proteins containing a portion of RARα and that are no longer responsive to physiological concentrations of RA. However, pharmacological doses of RA have made a major impact in the treatment of these patients. Finally, we examine the literature concerning the role of RARs and their level of expression in a variety of other tumor cells. In many, but not all cases, there is a loss in RARβ expression resulting in cells that are unresponsive to physiological doses of RA. In some cases, treatment with pharmacological doses of RA can be effective.

MECHANISM OF ACTION OF RETINOIC ACID

Within the past 15 years tremendous advances have been made in the understanding of the mechanism of action of RA (for review, see 12, 97, and references therein). Six nuclear receptors (termed RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ) have been cloned and extensively studied. Each of these nuclear receptors are encoded by distinct genes within the genome and are members of the steroid/thyroid hormone receptor superfamily. In vitro binding studies have demonstrated that the natural metabolites all-*trans*-RA (ATRA) and 9-*cis*-RA are high-affinity ligands for RARs, whereas only 9-*cis*-RA has been shown to bind RXRs.

RARs contain six functional domains termed A–F (Figure 1*A*). The amino terminal A/B domains are of variable length, the least conserved, and have been demonstrated to contain a ligand-independent transactivation activity (termed AF-1). The DNA-binding domain (domain C) contains a pair of zinc fingers that are responsible for making contacts with the major groove of DNA and for discrimination of the DNA response element half-site spacing. Domain D is known as the hinge region. In addition to binding ligand, the E domain also contains a ligand-dependent transactivation activity (termed AF-2) and accessory dimerization sequences. The function of the carboxyl terminal F domain has not yet been determined.

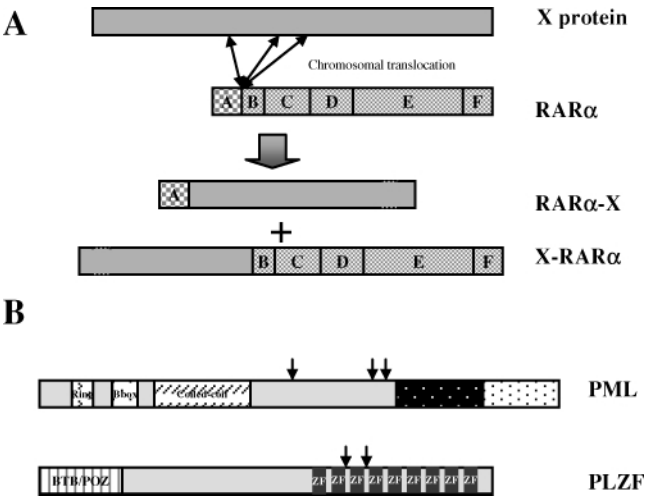

Figure 1 Chromosomal translocations in APL. (*A*) Chromosomal translocations generate fusion proteins between RARα and X protein. Although the break point in the gene that encodes X protein can be at various sites (indicated by arrows), the break point in RARα gene always results in the separation between A domain and B–F domains. (*B*) Chromosomal translocations result in the separation of PML and PLZF protein, two of the most common proteins that are found fused to RARα in APL. Arrows point to the reported separation positions in these two proteins. Abbreviations: APL, acute promyelocytic leukemia; RAR, retinoic acid receptor; PML, promyelocytic leukemia; PLZF, promyelocytic zinc finger.

These proteins, as heterodimers (RAR/RXR) or homodimers (RXR/RXR), function as RA-inducible transcriptional regulatory proteins by binding to DNA sequences called retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) located within the promoter of target genes. RAREs consist of direct repeats of the consensus half-site sequence AGGTCA separated most commonly by five nucleotides (DR-5), whereas RXREs are typically direct repeats of AGGTCA with one nucleotide spacing (DR-1). The RAR/RXR heterodimer binds to the RARE, with RXR occupying the 5′ upstream half-site and RAR occupying the 3′ downstream half-site.

Over the past several years a large number of proteins that interact with RARs have been demonstrated to play an important role in the ultimate control of their transcriptional activity (for review, see 36, 48, 102, 105, and references therein). In the absence of RA, the apo-receptor pair (RAR/RXR) binds to the RARE in the promoter of target genes and RAR recruits corepressors (Figure 2*A*). These corepressors mediate their negative transcriptional effects by recruiting histone deacetylase complexes (HDACs). HDACs remove acetyl groups from histone proteins. This induces a change in the chromatin structure, causing DNA to be inaccessible

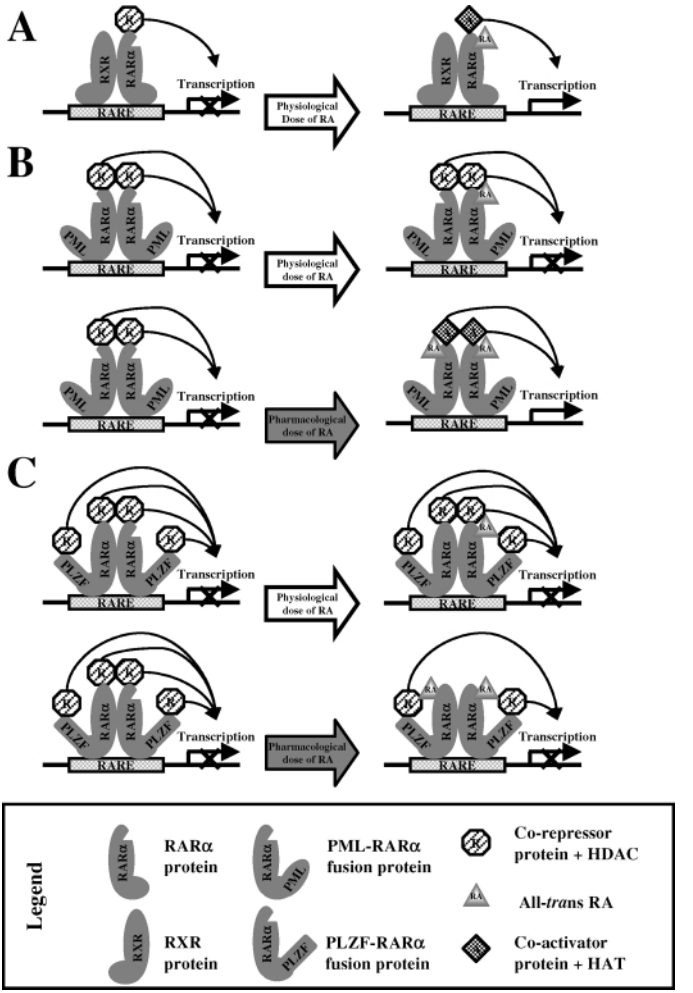

Figure 2 Molecular mechanism of leukemogenesis in acute promyelocytic leukemia. (*A*) Wild-type RARα, (*B*) PML-RARα, (*C*) PLZF-RARα.

to the transcriptional machinery. On the other hand, upon RA binding (at physiological levels) there is a conformational change in the structure of the ligand-binding domain that results in the release of the corepressor and the recruitment of coactivators to the AF-2 region of the receptor (Figure 2*A*). Some coactivators interact directly with the basal transcription machinery to enhance transcriptional activation while others encode histone acetyl transferase (HAT) activity. HAT acetylates histone proteins, causing the opening up of the chromatin and activation of transcription of the associated gene.

RARα AND ACUTE PROMYELOCYTIC LEUKEMIA

Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) was identified as a distinct subtype of acute myeloid leukemia (AML) in 1957 (43). It accounts for 8% to 15% of all AMLs, with a few thousand new cases of APL diagnosed each year in the world (29). In APL, a block in the myeloid differentiation program causes a large accumulation of abnormal promyelocytes that do not differentiate into mature, nonproliferating granulocytes in the bone marrow. Clinically, two features define APL: (*a*) abnormal promyelocytes in the bone marrow displaying a characteristic morphology that includes large granules and Auer rods (linear package of granules) (6), and (*b*) coagulopathy characterized by disseminated intravascular coagulation and fibrinolysis (4). Molecularly, all cases of APL are characterized by a nonrandom chromosomal translocation (or deletion in one case) that leads to the fusion of the RARα gene to one of five different partners (for review, see 118).

The clinical management of APL was very difficult due to the unpredictable onset of life-threatening bleeding, until 1988 when a group in Shanghai demonstrated the successful treatment of APL patients with ATRA (45). It is interesting to note that this finding preceded the demonstration of the involvement of RARα in APL. Today, unlike patients with other types of AML, greater than 80% of APL patients can obtain complete remission with a combination of chemotherapy and pharmacological doses of ATRA (34). Clinical studies have demonstrated that ATRA mediates this remission by terminal differentiation of the leukemic blast cells. This represents the first well-established clinical use of an oncogene-targeted differentiation therapy for the treatment of a specific cancer.

Translocations of RARα

The molecular pathogenesis of APL is one of the best understood of all malignancies (for review, see 72). All reported cases of APL involve the fusion of RARα to one of five different partners (designated X), resulting in the production of the fusion proteins X-RARα and RARα-X (Figure 1*A*). The X-RARα fusion protein is always expressed and is consistently implicated in the development of APL. On the other hand, the RARα-X fusion protein may or may not be detected. The molecular break point in the RARα gene is generally within intron 2; however, analysis of the genomic break points from a series of APL patients has demonstrated many different break points within this intron, including three significant microcluster break points (90). Although the mechanism of this translocation is not well understood, it is believed to be a result of nonhomologous repair and end joining of double-stranded DNA breaks (90). All X-RARα proteins contain the B to F domains of RARα, which include the DNA-binding domain, corepressor binding site, ligand-dependent transcriptional activation function (AF-2), dimerization interface, and the ligand-binding site of RARα within this fusion protein. On the other hand, the RARα-X proteins contain the

ligand-independent transcriptional activation function (AF-1) located in the A domain of RARα.

PML-RARα Greater than 98% of APL patients display a reciprocal and balanced translocation of the RARα gene on chromosome 17 to the promyelocytic leukemia (PML) gene on chromosome 15 t(15;17)(q22;q12-21) (9, 21, 66, 73). Five different PML-RARα fusion proteins have been described (78) (Figure 1*B*). These are determined by both the position of the break point in the PML gene (located in exon 3, exon 6, or intron 6) and alternative splicing of the PML portion of the fusion RNA. Despite these various isoforms of PML-RARα, many studies report excellent clinical outcomes in the majority of patients displaying the t(15;17) translocation upon treatment with ATRA (26). However, there are reports that cells expressing the PML-RARα isoforms derived from the break point located in exon 6 are less sensitive to ATRA treatment (31). The reciprocal RARα-PML proteins are expressed in approximately 80% of patients with this translocation (1). Expression of the reciprocal RARα-PML fusion protein is not obligatory for the development of APL in humans and appears to be unrelated to the clinical outcome of patients upon treatment with ATRA (62).

PML is a nuclear protein that contains a characteristic C_3HC_4 zinc-finger motif called the RING (really interesting gene) domain, located at the amino terminal end of the protein (87). It is followed by two additional zinc fingers (called B boxes) and an α-helical coiled-coil motif. The RING motif, B boxes, and coiled-coil motif are collectively called the RBCC domain. The RBCC domain is present in all PML-RARα isoforms regardless of the site of the break point in the PML gene (Figure 1*B*). It plays an important role in the dimerization and multimerization of PML-RARα but does not confer DNA-binding capability (47).

Wild-type PML, via the RBCC domain, localizes into large multiprotein nuclear structures called PML oncogenic domains (PODs) or PML-nuclear bodies (PML-NBs). More than 30 different proteins, including p53, pRB, DAXX, CBP, and eIF4E, have been shown to localize within these dense spherical structures; however, their exact function remains relatively unclear (47). PML appears to play a critical role in the formation and stability of the PML-NBs. Several recent reports have implicated PML as an important coordinator from the PML-NB of several tumor suppressor functions, including apoptosis, growth arrest, and cellular senescence (92). Interestingly, in APL cells expressing PML-RARα the normal structure of the PML-NBs is altered to a microspeckled nuclear pattern and RXR is delocalized from nuclear diffuse to a microspeckled pattern. After treatment with ATRA, the altered localization of RXR and the microspeckled pattern of the PML-NBs revert to normal (53, 103).

PLZF-RARα Approximately 1% of APL patients have a translocation between the RARα gene located on chromosome 17 and the promyelocytic zinc finger (PLZF) gene located on chromosome 11 t(11;17)(q23;q21) (14, 15, 33) (Figure 1*B*). Unlike patients with PML-RARα, patients with the PLZF-RARα translocation

generally are relatively insensitive to treatment with ATRA and have a poor response to chemotherapy (15). However, a few reports have recently indicated some success in the treatment of PLZF-RARα patients with ATRA along with other agents, including G-CSF (81).

PLZF is a nuclear protein that binds DNA in a sequence-specific manner via nine Krüppel-like C_2H_2 zinc fingers (15). It also contains a POZ (poxvirus and zinc finger) domain that mediates dimerization and transcriptional repression and can be involved in chromatin remodeling (13). PLZF target genes include homeobox genes, cyclins, and cell cycle regulators (118). One major PLZF-RARα isoform has been reported that contains the POZ domain and the first two zinc fingers of PLZF fused to the B through F domains of RARα. In addition, one case has been reported that contains the first three zinc fingers of PLZF (15) (Figure 1*B*). The reciprocal RARα-PLZF fusion protein contains the remaining seven (or six) zinc fingers along with the A domain of RARα, and can bind via the zinc fingers to PLZF target genes (59). However, instead of repressing their transcription like wild-type PLZF, transcription is activated due to the AF-1 domain of RARα and the loss of the POZ domain of PLZF (59).

NPM-RARα, NuMA-RARα, AND STAT5b-RARα Three additional very rare chromosomal alterations involving the RARα gene have also been described in APL patients. The first is the t(5;17)(q35;q21) translocation involving the nucleophosmin (NPM) gene (89), the second is the t(11;17)(q13;q21) translocation involving the nuclear mitotic apparatus (NuMA) protein gene (104), and the last is the t(17;17)(q11;q21) deletion involving the signal transducer and activator of transcription 5b (STAT5b) gene (3). The STAT5b-RARα fusion protein, unlike the other four APL-related fusion proteins that arise due to a chromosomal translocation, is a result of an interstitial deletion within chromosome 17. Hence there is no reciprocal fusion protein. APL patients with the NPM-RARα translocation and the NuMA-RARα translocation are sensitive to ATRA treatment, whereas the one reported patient expressing the STAT5b-RARα fusion protein is unresponsive to ATRA therapy (3).

Role of RARα Fusion Proteins in Leukemogenesis

The most definitive research demonstrating that the RARα fusion proteins are required for the development of APL has come from the study of transgenic mice expressing RARα fusion proteins with either PML or PLZF. Interestingly, the ubiquitous and unrestricted expression of PML-RARα results in embryonic lethality (42), whereas expression of PML-RARα in early hemopoietic progenitors or in mature myeloid cells does not result in leukemia (23). However, transgenic mice that express PML-RARα in the myeloid promyelocytic compartment acquire leukemia that resembles human APL, thereby demonstrating that PML-RARα is sufficient for the development of APL (11, 30, 42). The majority of these mice display a late onset of the leukemia, generally after 6 months of age (median of 8.5 months), and

an incomplete penetrance (64% of mice at 1 year), which suggests that additional genetic mutations are required for full transformation of the cells.

Other studies have demonstrated that expression of the reciprocal protein RARα-PML alone in transgenic mice does not result in APL, whereas coexpression studies involving both PML-RARα and RARα-PML suggest that RARα-PML can somehow modulate the phenotype in these transgenic mice. However, the exact function of RARα-PML remains unknown (40, 84). Finally, coexpression of PML-RARα with either BCL2 or interleukin 3 in transgenic mice has been demonstrated to be sufficient for the more rapid development of APL with 100% penetrance (52, 82).

It has therefore been suggested that the translocation between the RARα and PML gene resulting in the expression of the PML-RARα fusion protein may initiate leukemogenesis. However, additional genetic mutations may be required for the acquisition of acute leukemia. Recent studies have focused on the identification of potentially important additional chromosomal abnormalities in PML-RARα transgenic mice and APL patients that might cooperate with PML-RARα in leukemogenesis (52, 58, 94, 121). Future studies identifying the specific genes involved in these secondary chromosomal abnormalities will be useful in the identification of potential cooperating genes.

Similar studies designed to examine the role of PLZF-RARα in transgenic mice have demonstrated that both the PLZF-RARα and its reciprocal fusion protein RARα-PLZF are required for the development of APL. Transgenic mice expressing only PLZF-RARα develop chronic myeloid leukemia, whereas those expressing only RARα-PLZF do not develop any type of leukemia (40).

Molecular Mechanism of Leukemogenesis

How then do the various fusion proteins of RARα contribute to leukemogenesis? Because RARα is the common gene involved in all translocations associated with APL, the most likely explanation involves an interference with the normal function of RARα despite adequate levels of both vitamin A in the diet and RA in the cell. Many studies have clearly demonstrated that PML-RARα in the presence of physiological concentrations of RA functions as a dominant negative receptor, resulting in transcriptional repression.

PML-RARα displays altered DNA binding. It binds most often to RAREs as a homodimer (or oligomer) of itself rather than a heterodimer with RXR (80). The formation of PML-RARα homodimers or oligomers causes an enhanced corepressor binding efficiency. Consequently, the PML-RARα homodimer binds two corepressor molecules (and associated HDACs) with higher affinity compared with the one molecule of corepressor (and associated HDACs) bound by the wild-type RAR/RXR heterodimer (65).

Although both wild-type RARα and PML-RARα have a similar K_d for ATRA (5), at physiological concentrations of ATRA the corepressors (and associated HDACs) remain bound to PML-RARα homodimer, causing a repression of

transcriptional activity (65). However, pharmacological doses of ATRA are able to completely dissociate the corepressors (and associated HDACs) from PML-RARα homodimer, allowing the binding of coactivators and transcriptional activation (65). This explains why PML-RARα patients are unresponsive to physiological levels of ATRA, but are highly responsive to pharmacological doses of ATRA. Furthermore, this paradigm can be extended to explain the mechanism of RA responsiveness in APL patients that express NPM-RARα and NuMA-RARα fusion proteins (88) (Figure 2*B*).

On the other hand, patients with the PLZF-RARα translocation are not responsive to even pharmacological doses of ATRA. In this case both the RARα and PLZF portion of the PLZF-RARα fusion protein can bind corepressor and associated HDACs. Hence the homodimeric PLZF-RARα complex includes four molecules of corepressors (and associated HDACs). Like PML-RARα, PLZF-RARα has an affinity for RA similar to that of wild-type RARα, and the RARα-bound corepressors (and associated HDACs) are released by pharmacological doses of RA. However, the PLZF-bound corepressors (and associated HDACs) are not released upon binding of ATRA by the PLZF-RARα fusion protein. Therefore, even at pharmacological doses of ATRA, PLZF-RARα continues to function as a transcriptional repressor (41, 91). A similar mechanism has been demonstrated to explain the RA insensitivity of the patient expressing STAT5b-RARα (71) (Figure 2*C*).

At present there is little information identifying critical genes whose expression is altered by the X-RARα fusion proteins and that are responsible for the development of APL. Studies utilizing RAR knockout mice indicate that RARs are dispensable for normal hematopoiesis; however, in vitamin A–deficient mice RARs appear to play a modulatory role (for review, see 17). Therefore, it is currently unclear whether the X-RARα fusion proteins dominant negatively repress the expression of only genes normally regulated by RARα during neutrophil differentiation or whether the X-RARα fusion proteins also affect the expression of additional genes that lack a typical RARE. Recently, oligonucleotide array studies have identified 16 genes to be upregulated and 57 genes to be downregulated in cells expressing both PML-RARα and PLZF-RARα (79). In another study, ubiquitin-activating enzyme E1-like (UBE1L) expression was abolished in PML-RARα-expressing cells. Furthermore, UBE1L expression was found to be induced upon treatment with pharmacological doses of RA and to trigger the degradation of PML-RARα, suggesting the importance of this gene during leukemogenesis (51). Additional studies are necessary to determine the role of these genes in the development of APL and to identify additional potentially important genes. These studies would help in the understanding of the mechanism of APL and could lead to identifying new treatments for PLZF-RARα-induced APL. Other important areas that require additional investigation include the identification of the role of the reciprocal fusion proteins (X-RARα) during leukemogenesis and the determination of the role(s) of each of the partner proteins in both normal cells and APL cells.

Clinical Treatment

Although the vast majority of APL patients achieve clinical remission upon treatment with ATRA in combination with chemotherapy, approximately 30% of these patients will relapse. Most patients who relapse are or will soon become refractory to retreatment with ATRA (16, 25). Several reasons have been suggested to account for this acquired ATRA resistance, including (*a*) increased metabolism and clearance of RA due to induction of cytochrome P450 enzymes including CYP26, (*b*) increased cellular levels of cellular retinoic acid-binding proteins (CRABPs), and (*c*) selection of non-PML-RARα leukemic clones.

Up to 50% of patients who become unresponsive to ATRA treatment have developed additional mutations in the PML-RARα gene (22, 46, 70, 101, 120). These mutations localize particularly in the ligand-binding domain of the RARα portion of the fusion protein. Hence these mutant PML-RARα fusion proteins display reduced binding of ATRA and/or reduced RA-dependent transactivation activity, accounting for the inability of the patient to respond to ATRA treatment. Over the past several years a number of studies have demonstrated that HDAC inhibitors such as sodium butyrate and trichostatin A can enhance ATRA-induced differentiation of APL cells (64). Use of HDAC inhibitors may prove useful in the treatment of these relapse patients.

Finally, arsenic trioxide has been shown to be an effective treatment for many relapse patients with ATRA-resistant and chemotherapy-resistant APL. This treatment causes apoptosis rather than differentiation of leukemic cells. The mechanism underlying arsenic treatment appears to be mediated by multiple pathways, including targeting of the PML portion of the PML-RARα fusion protein.

RARs AND OTHER CANCERS

RARs and Inhibition of Cell Growth

In most RA-sensitive tumor cells, RA causes inhibition of cell growth. RA treatment reduces DNA synthesis, induces morphological changes, prolongs doubling time, and reduces colony formation in soft agar assays. This suppression of growth usually does not involve cell death of the tumor cells but rather their arrest in the G_1 phase of the cell cycle (109).

To determine the mechanism of RA-dependent growth inhibition of RA-sensitive tumor cells, many studies have focused on comparing the level of expression of RARs in large numbers of normal cells, RA-sensitive tumor cells, and RA-resistant tumor cells. RARβ mRNA and/or protein levels have been demonstrated to be reduced or absent in a variety of cancer cells, including breast (100), head and neck (113), lung (119), oral tissue (69), cervix (28), and ovary (111). Similarly, loss of RARβ expression has been observed in a variety of different tumor samples, including esophageal (86), prostate (68), breast (106, 114), and lung (83, 115).

There is a strong correlation between the ability of cancer cells to increase RARβ levels upon treatment with RA and RA-dependent growth inhibition. Tumors and cancer cells that are sensitive to RA treatment generally demonstrate an increase in the level of RARβ upon RA treatment, whereas those that are RA resistant fail to elevate RARβ levels (69, 100, 119). On the other hand, both RA-sensitive and RA-resistant tumor cells demonstrate an RA-dependent increase in transcriptional activity when cells are transfected with a RARE-dependent reporter DNA construct (100, 119). This strongly suggests that the failure to elevate RARβ levels upon RA treatment in RA-resistant tumor cells is not a general defect in the RA signaling pathway, but rather is a specific effect on the RARβ gene.

Overexpression of RARβ in a number of RA-resistant cancer cells restores the ability of these cells to inhibit growth upon treatment with RA (39, 60, 61, 93, 100, 111, 112, 119), whereas a reduction in RARβ levels by antisense techniques reduces the growth-inhibiting ability of head and neck squamous carcinoma cells (99) and ovarian carcinoma cells (111) upon RA treatment. Furthermore, studies in patients with premalignant oral lesions also demonstrate a correlation between the ability of RA to elevate RARβ levels and clinical outcome (67). Interestingly, only in ovarian tumor cells has it been reported that the overexpression of any RAR subtype (RARβ, RARα, or RARγ) will restore RA-dependent growth inhibition to RA-resistant cells (111). It is not clear in other tumor types whether RARβ expression is critical to restore RA sensitivity or whether the overexpression of any RAR subtype will be sufficient.

However, some reports do not demonstrate a correlation between RA-dependent increase in RARβ levels and growth inhibition in tumor cells. A study of 185 stage I non-small-cell lung carcinoma patient samples indicated that high levels of RARβ mRNA correlated with a worse clinical outcome (50), and examination of five head and neck squamous carcinoma cell lines indicated that loss of RARβ expression does not necessarily lead to loss of growth inhibition by RA (123).

Although the RARβ_2 and RARβ_4 isoforms are transcribed using the same RA-responsive promoter (termed P2), there is evidence that RARβ_2 functions as a tumor suppressor gene, whereas RARβ_4 acts as a dominant negative receptor. Transgenic mice that display reduced levels of RARβ_2 owing to the expression of an antisense RARβ_2 transgene have been shown to develop lung cancer (8). On the other hand, transgenic mice that express elevated levels of RARβ_4 have an increased incidence of hyperplasia and tumors in several tissues, including the lung and breast (7). Studies in F9 cells demonstrate that RARβ_2, but not RARβ_4, is necessary for RA-dependent growth inhibition of these cells (24, 55). Finally, studies in breast cancer cells also demonstrate that RARβ_2 is a potent inhibitor of cell proliferation, whereas RARβ_4 represses RARβ_2-mediated growth suppression upon RA treatment (37, 95).

Although there is strong evidence linking RARβ expression and tumorigenicity in a large number of different cancer cells and tumors, a number of reports suggest that the expression level of other RAR subtypes may also be important. In skin,

both RARα and RARγ were found to be downregulated during mouse skin tumor progression and carcinogenesis (20, 116). In estrogen-negative, RA-resistant breast cancer cells, Fontana and coworkers have reported that RARα levels are reduced and overexpression of RARα can restore RA-dependent growth inhibition (35). In addition, RARα expression correlated with RA-dependent growth inhibition in ovarian cancer cell lines (96, 110, 112), but not in ovarian tumors (49). Finally, RARγ expression was strongly correlated with retinoid-induced growth inhibition of squamous cell carcinomas from oral tissue (56) and the head and neck (54).

In conclusion, there is not a clear RAR requirement nor is there a single common mechanism of RAR action to explain RA-mediated growth inhibition of all tumor cells. However, loss of RARβ expression and RA-dependent induction of RARβ expression are most often observed in tumor cells, even in the presence of physiological levels of RA, and in tumors despite normal dietary intake of vitamin A. Microarray analysis has been employed in recent reports to identify genes whose level of expression are modulated by RA treatment of different cancer cells and tumors. One such report has identified several genes whose level of expression is altered shortly after RA treatment in an oral squamous carcinoma cell line (57). Additional microarray studies may help to identify early RAR target genes and help to elucidate common events that mediate RA-dependent growth inhibition of tumor cells.

Mechanism of RARβ Silencing

Although a loss in RARβ expression and RA-dependent induction of RARβ expression occurs in a variety of premalignant and malignant lesions, the mechanisms underlying this observation are less well understood. Unlike APL cells, which always contain a translocation involving the RARα gene, there is no clear alteration(s) (rearrangement or mutation) in the RARβ gene of tumor cells displaying a loss of expression of RARβ (27). A second mechanism that has been investigated to explain the silencing of RARβ expression is loss of heterozygosity of chromosome 3p24, the locus of the RARβ gene in humans. Although loss of heterozygosity on chromosome 3p24 has been described in many cancers, it has not been possible to demonstrate a correlation between loss of heterozygosity and RARβ expression (85).

Another possible mechanism that has been explored is the role of various other transcription factors in the regulation of RARβ expression (Figure 3). Two orphan receptors, nurr77 and COUP-TF, have been reported to be important in the regulation of the expression of RARβ in tumor cells (63, 108). In human lung cancer cell lines, COUP-TF is highly expressed in RA-sensitive cells, whereas a high level of expression of nurr77 is observed in RA-resistant cell lines. Overexpression of COUP-TF in RA-resistant breast cancer cells results in the restoration of RA-dependent increase in RARβ expression and RA-dependent growth inhibition. It has been suggested that COUP-TF binds to a DR-8 response element

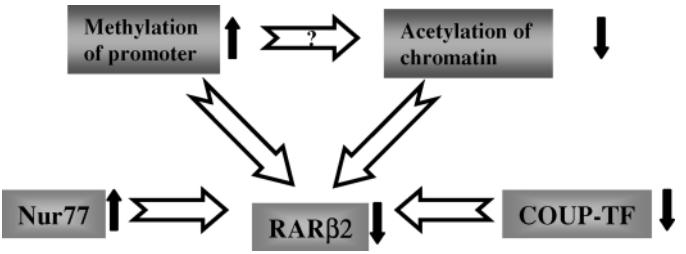

Figure 3 Potential mechanisms for silencing of RARβ2 in tumors. Small black arrows pointing up or down indicate increase or decrease, respectively.

in the RARβ P2 promoter in an RA-dependent and RARα-dependent fashion, causing an increase in the recruitment of CBP to the RARβ promoter by RARα (63).

Alternatively, Cote & Momparler have suggested that methylation is responsible for the lack of expression of RARβ in colon tumors (18) (Figure 3). Additional studies from this group have demonstrated that specific hypermethylation of cytosine resides in the region of -46 to $+251$ bp of the RARβ P2 promoter (10, 19). Since these initial reports many studies have confirmed an association between hypermethylation of the RARβ P2 promoter and either loss of basal or RA-inducible expression of RARβ in a large variety of cancer cell lines and primary tumors (2, 38, 75, 77, 107, 122). In addition, several studies have demonstrated that treatment of tumor cells with the demethylating agent 5-aza-2′-deoxycytidine can restore either the expression of RARβ or the inducibility of RARβ expression by RA (10, 18, 19). On the other hand, there are reports in which investigators fail to observe a correlation between the methylation status of the RARβ promoter and the expression of RARβ and/or are unable to demonstrate the reactivation of the expression of RARβ by treatment with demethylating agents (117).

Finally, several reports have demonstrated the reactivation of the expression of RARβ upon treatment of tumor cells with the histone acetylating agent trichostatin either alone or in conjunction with the demethylating agent 5-aza-2′-deoxycytidine (19) (Figure 3). A recent report has suggested that loss of histone H3 acetylation consistently correlated with RA resistance in lung cancer cells lines and loss of RARβ expression (98). DNA methylation was found to be involved in this aberrant histone H3 acetylation in some tumors cells, but not in others.

Taken together, both levels of specific orphan receptors and epigenetic changes to the RARβ gene (promoter methylation and histone deacetylation) have been demonstrated to contribute to the transcriptional silencing of RARβ2 despite the availability of physiological levels of RA in some, but not all, tumors cells. Demethylating agents and histone acetylating agents either alone or in combination with other agents, including RA, may be successful antineoplastic treatments, at least for some tumors.

CONCLUSIONS

Alterations in the level of expression or the functional activity of specific RARs is associated with a variety of types of cancer despite normal vitamin A nutrition. In APL, a translocation of the RARα gene in myeloid promyelocytes results in the expression of an abnormal X-RARα fusion protein in these cells that is responsible at least in part for the development of leukemia. Despite adequate dietary intake of vitamin A and physiological levels of RA in these cells, the X-RARα fusion protein functions as a transcriptional repressor rather than as a transcriptional activator. Only upon treatment of the patient with pharmacological doses of RA does PML-RARα but not PLZF-RARα function as a transcriptional activator. Hence, patients with PML-RARα can be successfully treated with pharmacological doses of RA.

In other cancer types, there is often a silencing of the RA-responsive RARβ gene in tumor cells that results in a lack of expression of RARβ despite physiological levels of RA. In some cases, pharmacological doses of RA, with or without DNA demethylating and histone acetylating agents, can reactivate the expression of RARβ, causing growth inhibition of the tumor cells.

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