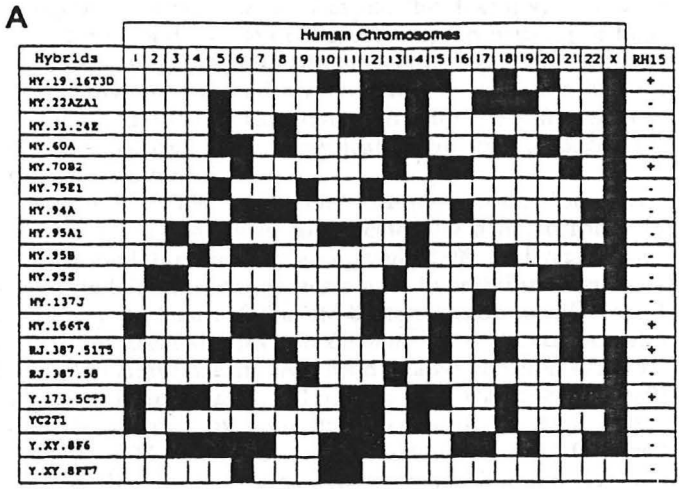
RETINOIDS:

"DIFFERENTIATION" AGENTS FOR CANCER TREATMENT AND PREVENTION



INTERNAL MEDICINE GRAND ROUNDS
JANUARY 23, 1992

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CASE PRESENTATION

P. B. is a 48 year-old woman who presented in July of 1988 with deep venous thrombosis and pulmonary embolism. A white cell count of 2.4 with 34% segmented neutrophils, 59% lymphocytes, 6% monocytes and 1% blasts was noted. Bone marrow examination was consistent with acute promyelocytic leukemia (French-American-British type M3). Cytogenetics revealed a balanced t(15;17) translocation.

She received standard induction chemotherapy consisting of daunorubicin 45 mg/m^2 IV for three days and cytosine arabinoside 100 mg/m^2 every 12 hours by continuous infusion for seven days. Despite this therapy, only a partial response was obtained and a second course of chemotherapy consisting of high dose cytosine arabinoside and L-asparaginase was administered. Again, a complete remission was not obtained. Finally, she entered a first complete remission 10/88 after treatment with mitoxantrone and VP-16. Consolidation with the same agents was given six weeks later. Her course was complicated by cryptococcal pneumonia and disseminated herpes zoster.

She relapsed in 9/89. A second complete remission was obtained with mitoxantrone and VP-16, and was complicated by a hemorrhage into the gluteal thigh muscle. Consolidation therapy was given 12/89. A liver biopsy, performed for persistently elevated liver function tests and hyperbilirubinemia, was consistent with primary biliary cirrhosis. Subacute bacterial endocarditis with Bacillus cereus was documented and treated with vancomycin. In 4/91, osteomyelitis of the ankle was treated with oral ciprofloxacin. A second relapse occurred 4/23/91.

Oral **all-trans retinoic acid,** 100 mg per day, was begun 5/6/91. A complete remission was documented by bone marrow biopsy 90 days later. Cytogenetics revealed that 100% of 30 metaphases were normal (46, XX). Oral retinoic acid was discontinued and the patient remained in remission for five months without further therapy.

Introduction

The purpose of this Internal Medicine Grand Rounds is to introduce a form of cancer therapy with an unusual mechanism of action--the selective induction of terminal differentiation of target malignant cells. For this discussion, I will concentrate on the use of all-trans retinoic acid (ATRA) for the treatment of acute promyelocytic leukemia (APL). The use of retinoids in the therapy and prevention of several squamous cell cancers will also be briefly discussed. We'll begin with a discussion of the pharmacology and metabolism of the retinoids.

The Retinoids: Derivatives of Vitamin A

The discovery of Vitamin A (all-trans retinol) in 1913 is credited to McCollum and Davis [1] as a result of dietary deficiency studies in rats. Rats fed a defined diet with lard as the sole source of fat developed a syndrome characterized by poor growth, failure to reproduce, and glandular atrophy which was reversed by the addition of an ether extract of butter. They called this extract "Fat Soluble A" to distinguish it from water-soluble nutrients ("Water-soluble B"). The pathologic lesion [2] was characterized by squamous metaplasia and keratinization of columnar epithelium.

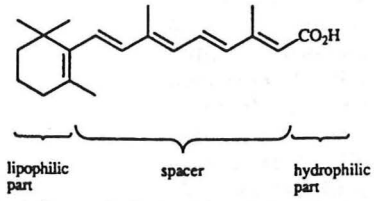
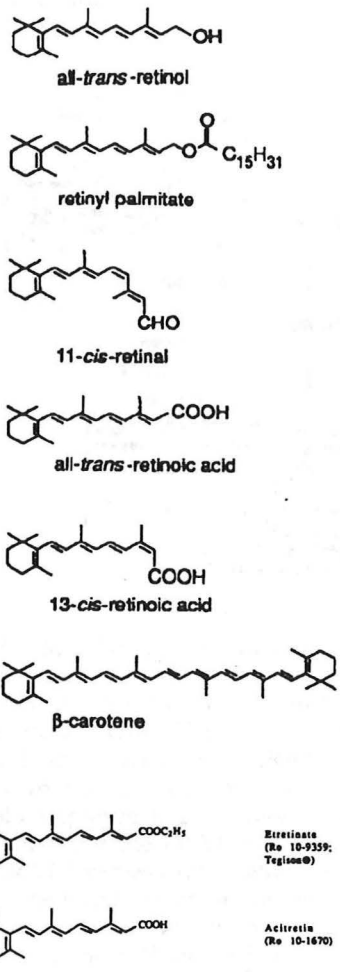


FIG. 1. Structural subunits of the retinoic acid molecule.

From ref. [4].

The structure of Vitamin A was determined in 1931 by Karrer, et. al. [3] and is presented in Fig. 1. The molecule has a lipophilic "head", a spacer region, and a hydrophilic "tail" [4]. Fig. 2 shows the structure of Vitamin A (all-trans-retinol) and some naturally-occurring derivatives, including retinyl palmitate (a dietary source from animal tissue), β-carotene (a tail-to-tail dimer of retinol and a major dietary source of provitamin A from plants), 11-cis-retinal (important in visual signal tranduction), and all-trans and 13-cis-retinoic acid (metabolites of Vitamin A generated within cells) [5]. Different retinoids selectively display some of the biologic activities of Vitamin A. For example, all-trans retinoic acid can support the growth and differentiation of epithelium, but not reproduction or vision (reviewed in [6]). Thus, animals maintained on retinoic acid in the absence of retinol are blind and sterile but otherwise healthy. Retinoids with therapeutic potiential include the naturally-occurring all-trans retinoic acid (tretinoin, Retin-A), 13-cis-retinoic acid (isotretinoin, Accutane) and chemically synthesized etretinate (Ro 10-9359, Tegison) and acitretin (Ro10-1670, Neotegison), which are used to treat psoriasis and other skin disorders [7].



Retinoids have a profound effect on cell differentiation and proliferation. They are essential to embryonic development, and synthetic retinoids are embryopathic or cause severe congenital malformations in humans and animals. Retinoic acid has been implicated as a morphogen in digital pattern development in the limb bud of the chick, where it induces or mimics the action of a "zone of polarizing activity", [8].. The most widely used *in vitro* functional assay system for retinoid action is the hamster trachea organ culture system developed by Sporn [9]. In this assay, the reversal of keratinization of Vitamin A-deficient trachea is measured. Retinoids also affect the differentiation of a variety of cultured cell lines . They induce terminal differentiation of mouse F9 teratocarcinoma cells and HL-60 promyelocytic leukemia cells in culture, affect the differentiation of keratinocytes and mesenchymal cells, and inhibit malignant transformation induced by chemical carcinogens [10], transforming growth factors, and phorbol esters. They act primarily by altering gene expression, as discussed below.

Fig. 2. Vitamin A and related compounds From ref. [5] and [7].

The Cellular Economy of Vitamin A

The transport and storage of Vitamin A has recently been reviewed ([5]) and is presented in Fig. 3. Retinyl palmitate from animal tissue is hydrolyzed to retinol by enzymes in the intestinal lumen while Vitamin A in the form of carotenoids are absorbed directly into enterocytes Carotenoids are partially converted through

retinaldehyde to retinol in the enterocyte by a tightly regulated process, so that ingestion of excessive amounts of β-carotene does not lead to clinical hypervitaminosis A [11].

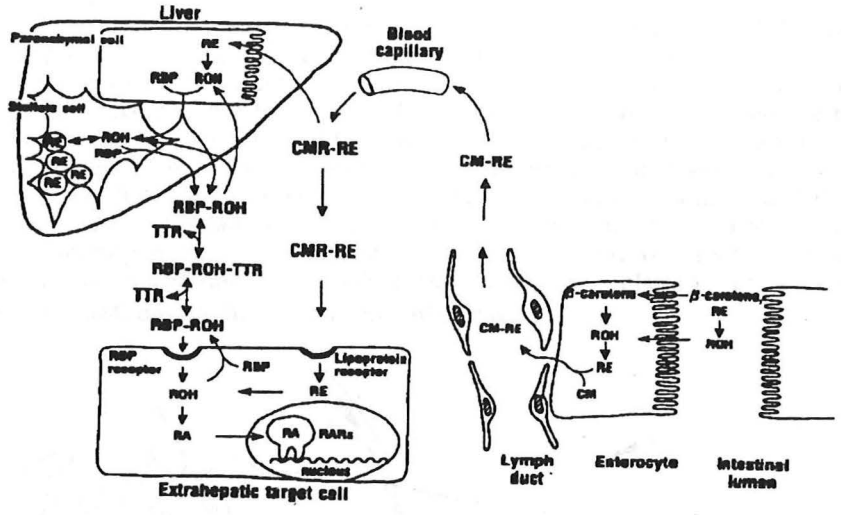


Fig. 3. Metabolism of Vitamin A. From ref. [5].

In the enterocyte, retinol is esterified by an acyl coenzyme A:retinol acyltranferase (ARAT) or by a lecithin coenzyme A:retinol acyltransferase (LRAT). ARAT is more active than LRAT, prefers membrane-bound retinol as its substrate, and is upregulated by large oral doses of retinol. LRAT is less active and prefers retinol complexed to an intracellular retinol binding protein (CRBP (II)). It has been postulated that the LRAT functions during the absorption of normal loads of retinol and ARAT handles excess retinol when CRBP II is saturated (reviewed in [5]).

The retinyl esters leave the enterocyte as a component of chylomicrons, which are absorbed into the lymphatics and eventually reach the systemic circulation. The association of retinol with chylomicrons has allowed lipid researchers to use Vitamin A as a marker for intestinal chylomicron production.

Chylomicrons are converted to chylomicron remnants in capillary beds mainly through the action of lipoprotein lipase; the retinol remains with the remnants. Chylomicron remnants are cleared primarily by the liver and to a lesser extent by bone marrow cells, spleen, and other extrahepatic tissues. The mechanism for cellular uptake is unclear at present but may involve an LDL-receptor-related protein (LRP) which binds to apolipoprotein E on the surface of apoE-rich chylomicron remnants [12].

In the liver, parenchymal cells take up the chylomicron remnant retinyl esters by receptor-mediated endocytosis, and free retinol is released from palmitate either at the plasma membrane or in an early endosome. The retinol does not seem to traffick to the lysosome; rather, it appears to be transferred directly to the endoplasmic reticulum to associate with a retinol-binding protein (RBP), which then mediates the secretion of retinol from the hepatic parenchymal cell.

Now here's the really interesting part. The retinol bound to RBP is taken up in a paracrine fashion by stellate (formerly known as "Ito") cells in the liver [13, 14]. These are perisinusoidal, myofibroblast-like cells which reside in the space of Disse and send processes that wrap around the endothelial cells which line the hepatic sinusoids (Fig. 4). Under conditions of Vitamin A loading, they accumulate large lipid droplets of pure retinyl esters. Other components of chylomicron remnants (Vitamin D, cholesterol esters) are not present. In mammals, up to about 80% of the total body pool of Vitamin A is stored in these cells in the liver. The stellate cells can apparently manufacture retinol binding protein and will secrete retinol-RBP directly into the plasma. The stellate cells thus control plasma retinol levels at a constant 2 μM, despite fluctuations in daily intake.

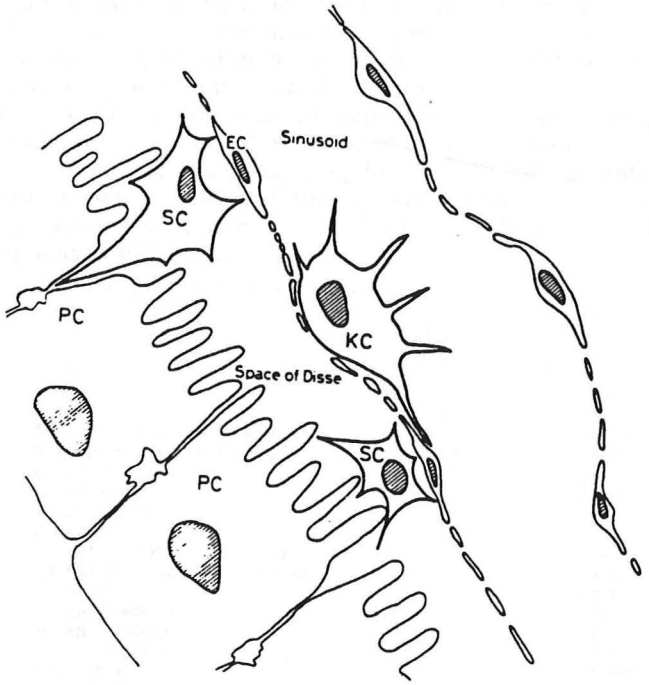


FIG. 4 Schematic diagram of the structure of the liver. PC, Parenchymal cell; EC, endothelial cell; KC, Kupffer cell; SC, stellate cell.

From ref. [13].

Most of the retinol-RBP is reversibly bound to a 55 kDa protein, transthyretin, which prevents glomerular filtration and urinary loss of the small retinol-RBP complex (21 kDa).

Target tissues take up retinol by mechanisms that are not understood. Possibilities include receptor RBP-mediated retinol uptake, nonspecific partitioning of free retinol or retinoic acid, fluid-phase endocytosis, or uptake through the chylomicron remnant receptor as retinyl esters.

Orally administered retinoic acid is absorbed directly into the portal system instead of the lymphatics, and circulates bound to albumin, by-passing the body's complex system for handling retinol [15]. The cellular uptake of retinoic acid, like that of retinol, is still a mystery.

Cellular retinoid binding proteins. A number of intracellular retinoid binding proteins have been described (Table 1, reproduced from [5]). CRBP(I) (cellular retinol binding protein I) is ubiquitous, with highest expression in liver, lung, kidneys, epididymis, and testes. CRBP(II) appears to be restricted to the absorptive cells in the intestine. Two cellular retinoic acid binding proteins (CRABP I and II) have also been described. CRABP I is ubiquitous and binds to all-trans and 13-cis retinoic acid with similar affinity, while CRABP II binds all-trans retinoic acid with much higher affinity. In the chicken, CRABP II expression is restricted to the developing skin, muscle, and bone of the embryo, and is expressed in a gradient which is inverse to the concentration of retinoic acid. Therefore, it may steepen the gradient of retinoic acid in the developing limb bud.

Some cell lines that have lost the CRABPs become unresponsive to retinoic acid. However, many cell lines do not appear to express any CRABP or CRBPs and yet respond to retinoids. Therefore, the function of cellular retinoid binding proteins is still unclear.

TABLE 1. *Retinoid-binding proteins and receptors*[a]

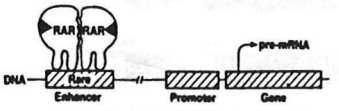
Protein	Approximate mass, kDa	Main ligand	Suggested function
RBP	21	Retinol	Blood plasma transport
IRBP	140	Retinol, retinal	Intercellular transport in visual cycle
Four proteins secreted from pig uterus	22	Retinol	Transport to the fetus
Two luminal proteins in rat epididymis	20	RA	Intercellular transport
CRBP (I)	16	Retinol	Donor for esterification, intracellular trar
CRBP (II)	16	Retinol	Donor for esterification
CRBP (III) from fish eye	15	Retinol	
CRABP (I)	16	RA	Intracellular transport, regulates free RA
CRABP (II) from neonatal rat	15	RA	Intracellular transport, regulates free RA
CRABP (II) from embryonal chick	16	RA	Intracellular transport, regulates free RA
CRALBP	36	Retinal	Enzymatic reactions in the visual cycle
RARα (7 isoforms)	48	RA	Ligand-dependent transcription factors
RARβ (3 isoforms)	48	RA	Ligand-dependent transcription factors
RARγ (7 isoforms)	48	RA	Ligand-dependent transcription factors
RXRα,β,γ	48	Unknown	Ligand-dependent transcription factors

Reproduced from ref. [5].

Metabolic conversion of retinol to retinoic acid. Once inside a target cell, retinol is oxidized to retinoic acid. It was long believed that the well-known enzyme, alcohol dehydrogenase (ADH), catalyzes this reaction, but a study has shown that an ADH-negative strain of deermouse is not defective in retinol oxidation [16]. Interestingly, a very recent study has shown that a minor isoform of ADH, called ADH III, is markedly upregulated by retinol feeding [17]. This is strong presumptive evidence that ADH III is the relevant converting enzyme. Retinoic acid may also be derived from β-carotene in several tissues [18]. Interestingly, few details are known about the production of retinoic acid in tissues and its regulation; this may be a fruitful area for future studies.

Nuclear retinoic acid receptors

How do retinoids mediate their effects on growth and differentiation? A large and growing body of evidence suggests that the nuclear retinoic acid receptors (RARs) mediate the classical effects of retinol on growth and development and differentiation in a manner analogous to the receptors for steroid hormones (i.e., the glucocorticoid, estrogen, progesterone and androgen receptors) and thyroid hormone, by activating gene expression (Fig. 5).



From ref. [5].

In fact, the first retinoic acid receptor (RARα) was identified from molecular cloning experiments in which a cDNA similar in sequence to the steroid hormone receptors was discovered [19, 20]. Expression of the cDNA produced a protein which did not bind steroid hormone, but which eventually was shown to bind retinoic acid. Furthermore, by creating hybrid molecules of the RAR and the DNA-binding portion of the glucocorticoid receptor, it was shown that the RAR was capable of activating gene transcription, even before its DNA target sequence was known. These experiments may be regarded as a kind of "reverse biochemistry", in which identification of gene products based on structure led rapidly to insights which were not immediately forthcoming by classical biochemical methods.

Two further isoforms of the retinoic acid receptor, RARβ and RARγ, have been identified on the basis of homology with RARα. Interestingly, RARβ was first identified as a nuclear receptor gene that was interrupted by a hepatitis insertion site in a hepatoma [21]. RARγ was cloned by low stringency hybridization [22-24]. All three isoforms activate target genes in the presence of low nanomolar concentrations of retinoic acid. RXRα, a fourth related receptor, identified by low stringency hybridization, activates target genes in vitro but at concentrations of retinoic acid in the micromolar range [25]. This receptor will be described in more detail below.

The primary amino acid sequences of all of the nuclear hormone receptors may be divided into six domains, as illustrated in Fig. 6. Domain C, the DNA binding domain, is 97% conserved among the three RAR isoforms and about 60% conserved among the various nuclear hormone receptors. This is the defining region of this class of nuclear transcription factors, and contains two "zinc finger" motifs. Domain E is the ligand binding domain and also functions to promote protein-protein interactions. The functions of the other domains of the RARs are unknown, but by analogy to other nuclear hormone receptors, one would expect that domain D would function in nuclear localization.

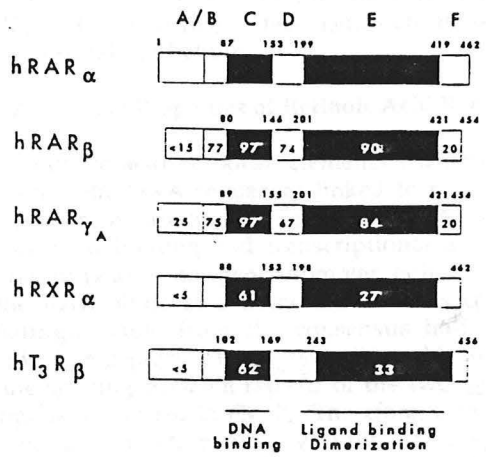


Fig. 6. Retinoic acid receptor sequence hormologies. From ref. [26].

Retinoic acid responsive genes

We have seen that induction of gene expression is a major mechanism for retinoic acid effects. But what are the target genes for the nuclear RAR? Three direct targets for which a retinoic acid-responsive DNA element have been demonstrated include the RARβ, ERA 1/Hox 1.6, and zif 268, which is a zinc finger protein (reviewed in [26]. All three of these targets are themselves DNA binding proteins which in turn regulate the transcription of other genes. Therefore, one may consider the retinoic acid receptor to be a master regulator, capable of beginning a cascade of gene transcription. Other attractive candiate target genes are the homeobox genes, which are important for segmental embryonic development. The Hox-2 cluster of homeobox genes is sequentially activated in a 3' to 5' direction in embryonal carcinoma cells by increasing amounts of RA. The relevant retinoic acid response elements in these genes have yet to be identified.

Not all genes directly activated by retinoic acid are themselves transcription factors. Another immediate target for retinoic acid is the alcohol dehydrogenase III gene [17]. As discussed above, ADH III converts retinol to retinaldehyde, the immediate precursor of retinoic acid, and it is positively regulated by retinoic acid in hepatocytes. The laminin B1 gene and mouse complement factor H gene have functional retinoic acid-responsive elements. Other genes which are not themselves transcription factors but which are responsive to RA include the EGF receptor, VIP receptor, and MSH receptor. Whether these genes have retinoic acid response elements is under investigation [26].

Response elements for the RXRα receptor have been identified in the genes encoding CRBPII [27] and apo AI [28]. These genes are involved in retinol uptake and metabolism in intestinal epithelium.

DNA Binding Properties of Retinoic Acid Receptors

By analyzing retinoic acid response elements (RAREs) in target genes and through the use of synthetic DNA sequences linked to reporter genes, it has been possible to define a consensus hexanucleotide sequence (A/G)G(T/G)TCA for RAR binding [26]. For optimal binding and transcriptional activation, the consensus sequence must be present twice in the gene promoter, either as a direct repeat or as a palindrome, and the RAR binds as a homodimer. This consensus "half-site" is, unexpectedly, indistinguishable from the consensus half sites for the thyroid, estrogen, and Vitamin D receptors! To oversimplify a bit, the explanation for this appears to be that the spacing between repeats of the two half-sites determines the specificity of binding, as illustrated in Fig. 7. Therefore, direct repeats separated by three random nucleotides function as a Vitamin D receptor recognition site, separated by four nucleotides as a thyroid hormone receptor binding site, and by five nucleotides as an RAR binding site. There is evidence that this similarity in half-sites allows for some "cross-talk" between these nuclear hormone receptor pathways!

The binding of RARs to DNA to directly activate transcription is only part of the story. Recently, it has been appreciated that nuclear "co-factors" increase the binding of RARs to DNA by more than an order of magnitude. (Similar cofactors have been described for other nuclear hormone receptors.) Protein cross-linking studies have indicated that these proteins enhance the binding by forming heterodimeric protein complexes with the RARs and that the proteins themselves, called RAR co-regulators, also bind to DNA. Most of these co-regulators have not been identified. An exciting recent development [29] has been the identification of a co-regulator, by screening of expression libraries with a RARE and RAR, as a protein related to RXRα, called RXRβ. RXRβ forms a heterodimer with the RAR to enhance its binding. It also forms heterodimers with the thyroid hormome receptor and the Vitamin D receptor to enhance the binding of these receptors to their cognate DNA binding sites as well. Remarkably, RXRa behaves similarly. Therefore, it appears that the "retinoid X receptors" are actually one class of cofactors that influences the

binding of several types of nuclear hormone receptors to their DNA recognition sites. Another mystery solved!

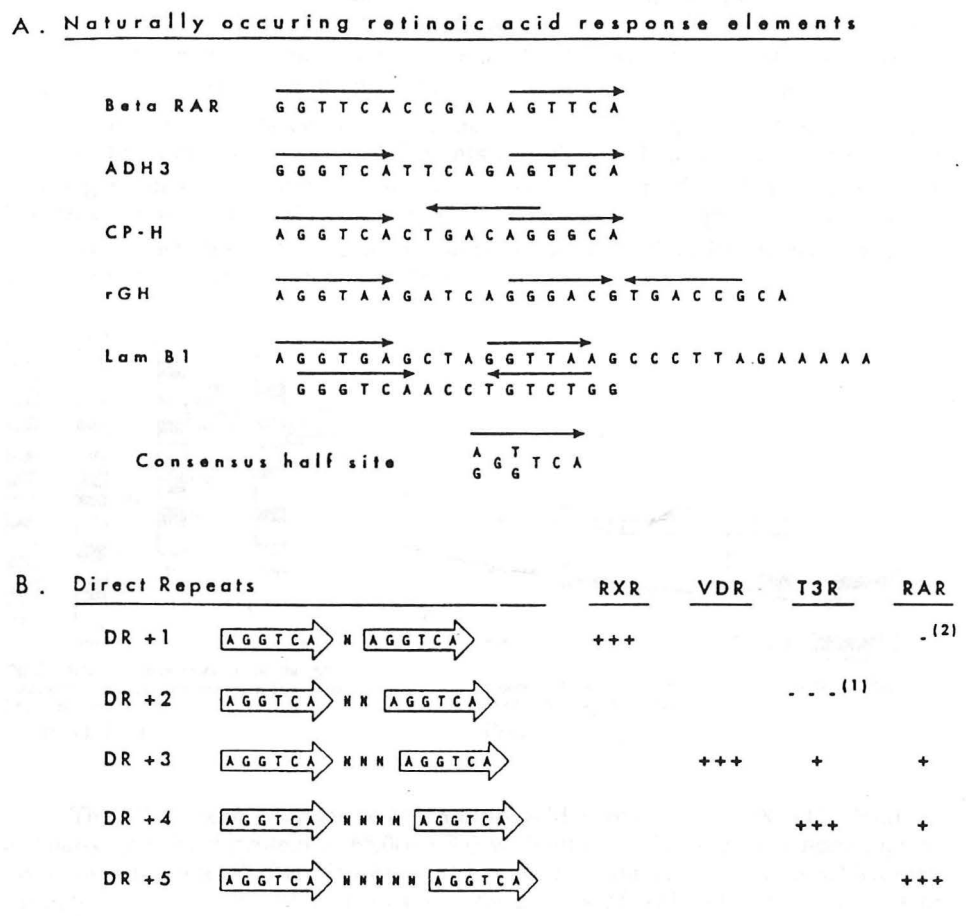


Fig. 7. Reproduced from [26].

The translocation breakpoint in acute promyelocytic leukemia

So why is retinoic acid useful in the treatment of acute promyelocytic leukemia? The answer lies in some stunning observations made recently by

investigators interested in the t(15;17) translocation breakpoint (Fig. 8), which is characteristic of APL [30-32]. These investigators found that the breakpoint involves the RARα on chromosome 17, band q21, and a myeloid-specific gene (initially called myl, later named PML, for promyelocytic leukemia) on chromosome 15. The translocation results in the synthesis of a PML/RARα fusion mRNA [33, 34], as shown in Fig. 9. The PML-RARα mRNA encodes a protein containing most of the PML sequences and a large portion of the RARα, including its DNA and hormone binding domains. The DNA breakpoints are clustered in the first intron of the RARα gene in APL patients, so that an invariant portion of the RAR is expressed. The breakpoint in the PML gene is also clustered, but not as precisely, resulting in two different lengths of PML protein contributing to the PML-RARα fusion protein, in the handful of patients looked at thus far.

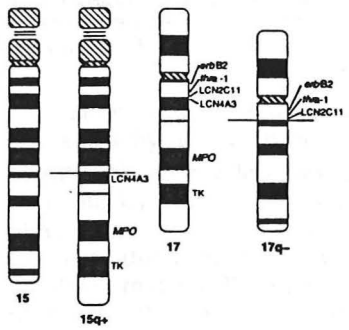


Fig. 8. Schematic representation of the APL translocation showing the derivative 15q+ and 17q− chromosomes.
From ref. [31].

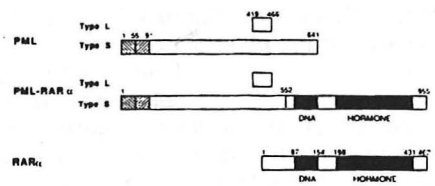


Figure 9. Physical Maps of PML, RARα, and PML–RARα cDNAs Isolated from the t(15;17)-Carrying Cell Line NB4
From ref. [34]

The PML gene encodes a 641 amino acid protein of 70,000 kDa (and an alternatively spliced protein of 65,000 kDa) without extensive sequence homology to any known protein [33, 34]. However, it has several tantalizing features which may ultimately prove to be relevant to the pathogenesis of APL (Fig. 10). Clusters of cysteines at the amino terminus fall into a metal-binding motif (distinct from "zinc fingers") found in several DNA binding proteins. Of note is that one of these homologous proteins, RAG-1, is a recombination-activating gene product in yeast involved in repair of UV-induced DNA damage. A proline-rich N-terminal region is characteristic of the transactivation domains of mammalian transcription factors. A leucine-zipper motif, which mediates protein-protein interaction between transcription factors, is also present.

It is interesting that the 5% of patients with APL who lack the PML-RARα transcript are precisely those patients who do not respond to retinoic acid (see below). Therefore, the abnormal transcript is strongly implicated in the pathogenesis of APL and its responsiveness to retinoic acid.

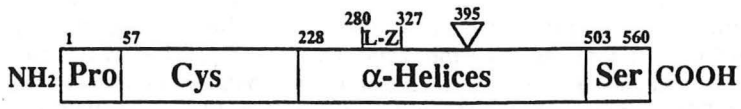


Figure 10 Nucleotide and Amino Acid Sequence of *PML-1* cDNA Isolated from an APL Patient mRNA

(a) The triangle represents the fusion point seen in *PML–RAR*. A potential polyadenylation signal is underlined. Both ATGs are shown in bold type (nucleotides 81–83 and 147–149).

(b) Schematic structure of the PML-1 protein. The encoded protein contains four regions of interesting structure. They are denoted as follows: Pro, proline-rich region; Cys, cysteine-rich region; α-Helices, α helix–rich region; Ser, C-terminus region where several serines are surrounded by prolines. The triangle shows the fusion point seen in *PML–RAR*. L-Z shows the position of a putative leucine zipper. The numbers above the boxes represent the amino acids at the junction of the assigned regions.

From ref. [33].

How does the PML-RARα fusion protein contribute to the development of APL, and why do high doses of all-trans retinoic acid overcome its effect and allow the APL cells to develop normally? The simplest explanation is that the PML-RARα acts to block myelocyte differentiation (reviewed in [35]. It could do this in two ways--either by acting as an RAR antagonist, blocking the normal effects of retinoic acid on myelocyte differention, or by acting as a PML antagonist. In the latter case, one postulates that the PML gene is needed for normal myelocyte differentiation. In either case, the abnormal portion of the protein is hypothesized to interfere in a dominant negative fashion with the normal functioning of the other. Retinoic acid would then act to relieve the inhibition. Note that both PML and RARα have dimerization domains which could mediate interactions with other transcription factors, an observation entirely consistent with the dominant negative hypothesis.

Does the PML-RARα interact in an altered way with retinoic acid response elements? The answer appears to be yes, but the direction of the response is a matter of debate. One group, led by Ron Evans in LaJolla, CA [34], finds that PML-RARα is a superinducer of reporter genes linked to the retinoic acid response element, while the French group [33] finds that it is an inhibitor. Both groups have traded reagents and can replicate the others' findings. However, they work in different systems, with different reporter genes, cell types, and promoters. Obviously, more work is needed to sort out these discrepant results. Ron Evans favors the hypothesis that interference with the normal PML function is the mode of action of the PML-RARα, and that the binding of the abnormal transcript to retinoic acid response elements is irrelevant to the leukemia. Both groups are looking at targets genes activated by PML and RARα, and are collaborating on efforts to transplant bone marrow cells that express PML and PML-RARα into mice to look at effects on myeloid differentiation.

Early studies. In 1980, Breitman, Selonick and Collins [36] reported that the naturally occurring isomer all-trans retinoic acid (ATRA) induces the differentiation of HL-60 cells in culture. HL-60 cells were derived from a patient with APL and maintained in continuous culture three years earlier. They assessed differentiation morphologically and by the ability to reduce nitroblue tetrazolium (a differentiated function of neutrophils) at concentrations as low as 1 nM. ATRA was also tested against freshly isolated leukemic cells in suspension culture. Of 21 leukemias, only two, both from APL patients, differentiated in response to ATRA [37]. Oddly, they reported that the 13-cis isomer was as effective as the all-trans isomer [36]. This led to the treatment of several small series of patients with 13-cis RA (which was easily available as Accutane, already in use for the treatment of acne) with only modest success [38-41].

Fortunately, a group from Shanghai Second Medical College in China went to the trouble and expense of making all-trans retinoic acid in large enough quantities for a clinical trial, and in 1988 in a paper in Blood [42] reported that 23 of 24 patients, 16 newly-diagnosed and eight who were resistant to previous chemotherapy, had achieved complete hematologic remissions on oral ATRA alone. The patients had all been induced as outpatients and the only side effects were dry skin and mucous membranes and occasional headache. Importantly, none of the patients experienced the coagulopathy associated with remission induction of APL by standard cytotoxic chemotherapy, which normally kills 10-20% of such patients. The patients received 45-100 mg/m^2/d and were changed to 20-30 mg/m^2/d after CR was obtained, which occurred at a median of 47 days. With a mean follow-up of 11 months, eight of the 23 patients relapsed on the lower dose of ATRA, which is comparable to results seen with standard chemotherapy (overall long-term CR rate of 25% [43])

Understandably, this report was met with initial skepicism. However, three recently published trials, one French, one American and one Chinese, have borne out their observations, and the salient features of these studies will be described in more detail below.

The French Experience--Hopital St. Louis, Paris. [44]. This study involved 22 patients, most of whom were previously treated with and had failed conventional therapy (18); the remainder had contraindications to conventional chemotherapy (extreme age, poor cardiopulmonary function, previous extensive radiation therapy). The dose of ATRA was 45 mg/m^2/d orally for 90 days. After 90 days, most patients received maintenance chemotherapy consisting of either daily 6-mercaptopurine (100 mg/m^2) and weekly methotrexate (12 mg/m^2). Two patients each received either weekly mitoguazone (200 mg/m^2) and Ara-C (100 mg/m^2) or continued retinoic acid. The time course of the leukocyte response to ATRA is shown in Fig. 11. As in the initial Chinese study, there was a high incidence of complete responders. Only one patient failed to respond at all; this patient's cells

did contain the t(15;17) translocation but the cells were resistant in vivo and in vitro. Another three patients had initial complete cytogenetic response but relapsed while still on the drug and were considered "transient responses". Three other patients developed a previously undescribed hyperleukocytosis syndrome, with acute cardiorespiratory failure and leukocyte counts of 73,000, 80,000 and 32,000 just before death at six, eight, and 12 days after treatment. The remainder achieved CR. All had cytogenetic studies which failed to detect the t(15;17) translocation. Somewhat disappointingly, but not surprisingly, most patients eventually relapsed, including the two patients who received continued therapy with ATRA. Five patients were still in CR at a median follow-up of seven months at the time of the report.

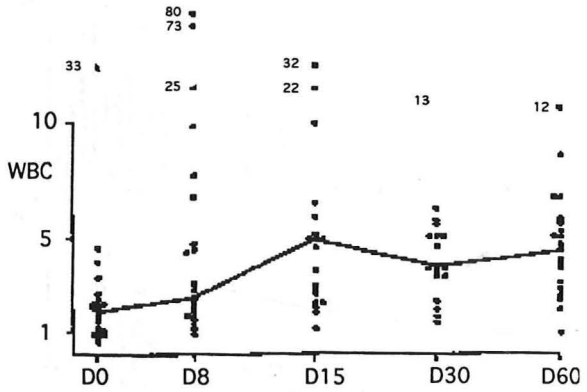


Fig. 11. Leukocyte responses in patients treated with ATRA. From ref. [44].

To summarize, this study illustrated the following:

1. Complete remissions could be obtained with rapid correction of DIC, no bone marrow aplasia, and treatment on an outpatient basis with minimal need for transfusions and other supportive care.
2. The duration of CR was short, indicating that the drug does not completely eradicate the leukemic clone. However, normal 46,XX or 46,XY hematopoiesis was established in these patients, indicating that RA induces terminal differentiation, and eventual death of the leukemic cells.
3. Several patients who had somewhat high initial wbc counts (recall that APL usually presents with low wbc counts) developed a fatal hyperleukosis syndrome. More about this below.

In a companion paper [45], *in vitro* studies on the leukemic cells of these patients were reported. In contrast to the earlier *in vitro* study, they found a ten-fold

difference in the efficacy between all-trans vs. 13-cis RA, with maximal results at 10^{-7} M with ATRA and 10^{-6} M with 13-cis RA (see Fig. 12). This observation explains the previous poor results in APL with Accutane.

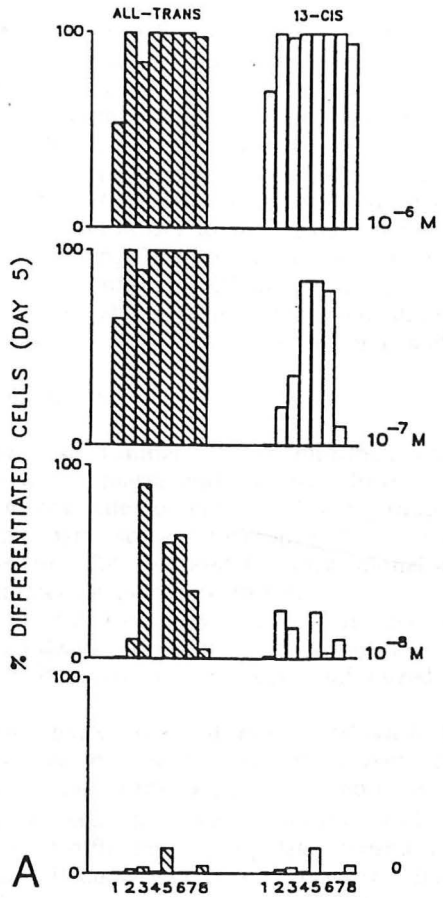


Fig 12 Structure-activity studies of all-trans RA and 13-cis RA.

From ref. [45].

A more recent study from the French which looked only at newly-diagnosed patients was presented at the American Society of Hematology meetings in Denver recently, and is published in abstract form [46]. Recognizing the need for definitive further therapy in these patients, patients received conventional chemotherapy with daunorubicin and Ara-C as soon as CR was achieved. Twenty-six of 27 patients

achieved CR, in 35 to 80 days after initiation of therapy. At a median follow-up of 11 months, only two patients relapsed, one of whom never received the conventional induction therapy. Four patients developed the hyperleukocytosis syndrome.

The Chinese Experience-Suzhou Medical College, Jiangsu. This was a large (50 patients) trial of previously untreated patients (47) [47]. The patients received ATRA, 60-80 mg/d until CR was achieved (as defined by the reduction of blasts plus promyelocytes in bone marrow to less than 5%). Again, the vast majority (94%) achieved CR. In this study, all patients then received conventional induction chemotherapy; eight patients also received continued low-dose retinoic acid. It was not clear how these patients were selected. Interestingly, all eight of these patients eventually became refractory to any further therapy, including ATRA, and all eight died. Of the remaining 39 who did not receive continued ATRA, only 11 relapsed, and the rest are in continuous CR at a mean follow-up of 15 months. Interestingly, most of the patients who relapsed (8/11) were still sensitive to RA and are alive and in CR after repeating the cycle at six, four, three, and seven months of follow-up.

To summarize:

1. The high rate of initial CR was confirmed, again with quick control of DIC, no bone marrow aplasia, and no hospitalization.
2. Conventional chemotherapy following induction of CR by ATRA was met with reasonable success (Overall, 60% in continuous CR at 15 months).
3. Relapse after RA followed by conventional induction chemotherapy did not predict later unresponsive to RA.
4. They concluded that low-dose RA as "maintenance" therapy was uniformly bad (8/8 dead). The conclusion would have been stronger if they had stated whether these patients had been randomized to this treatment.

The American Experience--Memorial Sloan-Kettering. The U.S. has lagged somewhat behind in studying ATRA in APL, partly because of the its later availability here. One study [48], from Memorial-Sloan Kettering, treated 11 patients, six newly diagnosed and five in relapse, with 45 $mg/m^2/d$. The drug was formulated a little differently (soft gelatin capsules) and given in two divided doses six hours apart. Patients with newly-diagnosed disease received ATRA for 30 days after CR followed by conventional induction therapy. The remainder received either continued ATRA or bone marrow transplantation.

Two patients failed to obtain a CR. One of these did not have the t(15;17) translocation at diagnosis and no aberrant PML-RARα transcript was detected. The other patient had only a partial response. The study confirmed the ease of obtaining a CR in the majority of patients. Four patients developed leukocytosis; the management of which will be discussed in more detail below. Follow-up was too short to assess long-term response.

In this paper, nine of the patients had molecular analysis of the PML-RARα transcript during the course of treatment. One patient, who lacked the t(15;17) translocation and did not have the aberrant transcript, was completely unresponsive to ATRA. One patient who lacked the translocation but did express the PML-RARα transcript responded well. As patients entered CR, the aberrant transcript decreased markedly and disappeared in some patients but not in others, including one who relapsed early. Thus, the abnormal transcript will undoubtedly be a useful marker for minimal residual disease in APL, in an analogous fashion to the bcr-abl transcript in chronic myelogenous leukemia (CML).

Side effects of therapy--the hyperleukocytosis syndrome and symptoms of hypervitaminosis A. In the French [44] and American [48] studies described above, several patients developed a syndrome of respiratory distress, fever, pulmonary infiltrates, pleural/pericardial effusions, edema, and impaired myocardial contractility within 5-15 days of beginnning ATRA for APL. The course of four such patients is presented in Fig. 13. In all but one case, the syndrome was associated with hyperleukocytosis with mature-appearing neutrophils (wbc's 30,000 to 80,000). Autopsy of two patients showed pulmonary infiltration with mature leukocytes, even in a patient with a wbc count under 10,000.

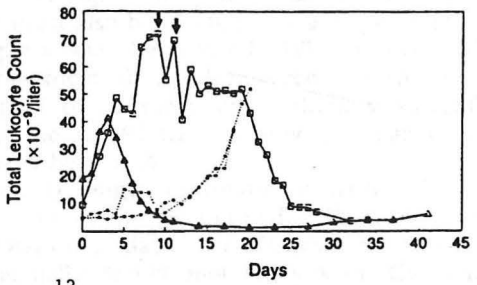


Figure 13. Development and Course of Peripheral Leukocytosis in Four Patients with Acute Promyelocytic Leukemia during Treatment with Tretinoin. From ref. [49].

At a recent meeting, the Memorial Sloan-Kettering group reported an incidence as high as 9/35 patients (26%--as compared to 4/27 or 15% in the French study). They obtained prompt resolution of these signs and symptoms with dexamethasone 10 mg IV b.i.d. for three days. Presumably, the steroids either inhibit leukocyte migration or antagonize the effects of cytokines. Leukapheresis was also helpful in several cases but may be unnecessary. The bias of the American group is that the use of standard induction chemotherapy in the face of this syndrome exacerbates the problem, resulting in DIC and tumor lysis syndrome. The French deal with it by treating early with chemotherapy if the leukocyte count increases by more than a certain amount at defined times early in therapy (>6000 on day five or >10,000 on day 15). A more reasonable plan may be to temporarily interrupt ATRA, institute

dexamethasone, and consider leukapheresis if there is not a prompt response to the corticosteroids.

Headache and papilledema (pseudotumor cerebri), which are symptoms of hypervitaminosis A, occurred in a couple of patients in one series [48], but did not necessitate changing the dose of medication. In one case, it was treated by intermittent lumbar puncture.

Transient severe bone pain was reported in about a third of the French patients but in none of the patients in the other studies. The French also observed transient elevations of the SGOT to 2-10 times normal. Hypertriglyceridemia was common, but was asymptomatic and resolved on cessation of therapy. All patients experienced a tolerable degree of dry mouth and mucous membranes and dry eyes.

Summary: Defining the optimal use of ATRA in APL

The studies discussed above demonstrate that complete remissions are obtainable with ATRA alone in the vast majority of patients with APL, whether the patients are newly-diagnosed or previously treated with conventional chemotherapy. Unfortunately, the duration of remission, even during continuous ATRA therapy, is significantly less than a year. Therefore, standard induction chemotherapy must also be given, but it is hoped that with ATRA, early deaths due to DIC and aplasia may be avoided. Whether these advantages will outweigh the risk of hyperleukocytosis syndrome remains to be seen. A large, prospective, randomized trial of standard chemotherapy vs. ATRA followed by standard chemotherapy, the APL91 trial, is now in progress in Europe. The NCI is also sponsoring a trial here in the U.S.

The optimal length of treatment with ATRA before standard induction chemotherapy has yet to be defined. The Memorial Sloan-Kettering group is continuing ATRA for 30 days after CR, while the French allow for no waiting period after CR, and actually start chemotherapy before CR in many patients, because of fear of the hyperleukosis syndrome. As discussed above, this approach may actually be undesirable, since the syndrome can be readily managed with corticosteroids.

Another interesting consideration concerns the development of "resistance" to ATRA in the patients who relapse. Warrell, et. al. [49] presented data at the recent American Society of Hematology meetings that there is a progressive decline in the plasma levels of ATRA during continuous treatment, to less than half of initial steady-state levels The leukemic cells actually retain their sensitivity to ATRA in vivo. Unfortunately, doubling the dose of ATRA did not significantly raise the plasma levels. Apparently, the homeostatic mechanisms regulating the cellular metabolism of Vitamin A and its metabolites, discussed in detail above, eventually operate to decrease the levels of retinoic acid available therapeutically. It is hoped that our advanced knowledge of these mechanisms will lead to its optimal use, and its full therapeutic potential will be realized in the future.

Future directions: Use of retinoids in other malignancies

Cancer therapy. Clinical investigations into the use of retinoids has been limited to retinol and retinyl esters, all-trans retinoic acid, and 13-cis retinoic acid. Most studies have used 13-cis retinoic acid because early studies suggested a greater therapeutic index. In light of the APL experience, this may have to be re-examined. To summarize a large body of data (reviewed in [50]), retinoids have been tested in small numbers of patients with a wide variety of tumors with minimal response, with the exception of basal cell and squamous cell carcinomas of the skin, and mycosis fungoides. Interestingly, retinoids accumulate to high levels in the skin. Complete responses have been seen in about 50% of basal cell cancers, and close to that in squamous cell cancers, but the responses are transient, with relapses occurring within one to two months after cessation of therapy. I suppose that there may be a place for retinoids in shrinking tumors prior to definitive therapy, in an analogous fashion to APL discussed above. Close to a dozen small studies of 13-cis retinoic acid and etretinate in mycosis fungoides have been published, with a complete response rates around 10 to 40%, and occasional "miraculous" responses in otherwise refractory patients [50]. As in other studies, relapse occurs rapidly after the drug is discontinued. Its role in this disease is an area of active investigation.

Chemoprevention. Since deficiency of Vitamin A leads to hyperkeratosis of the skin and squamous metaplasia of mucous membranes and the tracheobronchial tree, it is logical that malignancies which are preceded by squamous metaplasia have been targeted for chemoprevention trials with Vitamin A derivatives. The chemopreventive effect of retinoids has been studied extensively in animals (reviewed in [51] and in [52]). Protective effects are seen in skin, lung, mammary gland and bladder. These initial encouraging results have prompted several chemoprevention trials in patients at high risk of malignancy--those with premalignant skin lesions, including xeroderma pigmentosum [53]; oral leukoplakia [54]; and patients with treated primary ENT or lung tumors [55].

In 1988, Kraemer, et. al. [53] conducted a three-year controlled prospective study of 13-cis RA (2 mg/kg/d for two years) in five patients with xeroderma pigmentosum. These patients have an extremely rare, autosomal recessive deficiency in the repair of UV-induced DNA damage. In the two years before treatment, the patients had a total of 121 tumors (mean, 24, range, eight to 43). During treatment, they had 25 tumors (mean 5, range, 3 to 9). The tumor frequency returned to baseline after the drug was stopped. Interestingly, the effect was rapid, turning off tumor appearance within two months of the start of therapy, and quickly losing effectiveness after it was stopped. This indicates that the drug acts at a relatively late stage in tumorigenesis. Partly as a result of this positive trial, numerous clinical trials in other cutaneous "premalignancies" are in progress.

Patients with head and neck malignancies who receive effective local treatment are at high risk of recurrence both from the original primary and from new primary tumors. Hong, et. al. [55] randomized 103 patients to receive either 13-cis RA (50 to 100 mg/m2/d) or placebo for one year following definitive therapy. No

effect on recurrence of the primary was seen, but with a median follow-up of 32 months, only 2 patients (4%) had second primaries in the treated group vs. 12 (24%) in the placebo group. The second primaries were all head and neck, lung and esophagus. Unfortunately, 33% of the patients had to discontinue the drug during the one year of treatment because of intolerable skin dryness, conjunctivitis, cheilitis, and hypertriglyceridemia. Further primary chemoprevention studies of head and neck cancers and lung cancer are either planned or in progress, but the need for an effective retinoid with a greater therapeutic index is obvious. Over 2000 retinoids have been chemically synthesized [50], and perhaps such a compound will be forthcoming.

The vitamin A precursor, β-carotene, is virtually without toxic effects except for skin discoloration; as discussed above, limted metabolic conversion protects the body against hypervitaminosis A from this source. Indeed, there is some (albeit, controversial) epidemiological evidence linking low plasma levels of carotenoids with lung cancer (reviewed in [56]). One large prospective trial of β-carotene in patients with a single nonmelanoma skin cancer, conducted over five years, failed to show benefit [57]. Of course, β-carotene would not be expected to produce high levels of active retinoids in the skin. A randomized primary prevention trial of b-carotene in 20,000 physicians is in progress as part of the Hennekens study (the same study which showed a benefit for aspirin) and we must eagerly await the results [58].

Conclusion

Although a prospective randomized trial of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia has yet to be completed, I believe that ATRA will ultimately prove to be very valuable in the treatment of this disease, for remission induction prior to definitive chemotherapy. Since the relative efficacy of all-trans over 13-cis retinoic acid was a surprise in this disease, the role of ATRA in other serious malignancies will need to be re-examined. Effective use of retinoids in chemoprevention will depend on the development of less toxic drugs.

Finally, retinoic acid was found to be effective in APL several months before the RARα gene rearrangement was implicated in the disease (clinicians 1; basic scientists 0). This should not discourage basic scientists from examining every available tumor type for rearrangements in nuclear hormone receptor genes (of which there are many) with the hope and expectation that surprising new therapies for cancer will result.

References

1. McCollum, E.V. and M. Davis, *The necessity of certain lipids in the diet during growth.* Journal of Biological Chemistry, 1913. **15**: p. 167-175.

2. Wolbach, S.B. and P. Howe, *Tissue changes following deprivation of rat-soluble A vitamin.* Journal of Experimental Medicine, 1925. **42**: p. 753-777.

3. Karrer, P., R. Morf, and K. Schopp, *Zur kenntnis des Vitamin A aus Fischtranen.* Helvetica Chimica Acta, 1931. **14**: p. 1038-1040.

4. Klaus, M., *Structure characteristics of natural and synthetic retinoids.* Methods in enzymology, 1990. **189**: p. 3-14.

5. Blomhoff, R., *et al., Transport and storage of Vitamin A.* Science, 1990. **250**: p. 399-404.

6. Goodman, D.S., *Vitamin A and retinoids in health and disease.* New England Journal of Medicine, 1984. **310**: p. 1023-1031.

7. Boyd, A.S., *An overview of the retinoids.* American Journal of Medicine, 1989. **86**: p. 568-574.

8. Wedden, S., C. Thaller, and G. Eichele, *Targeted slow-release of retinoids into chick embryos.* Methods in Enzymology, 1990. **190B**: p. 201-209.

9. Sporn, M.B. and D.L. Newton, *Retinoids and chemoprevention of cancer,* in *Inhibition of tumor induction and development,* M.S. Zedeck and M. Lipkin, Editor. 1981, Plemun: New York. p. 71-100.

10. Lotan, R., D. Lotan, and P.G. Sacks, *Inhibition of tumor cell growth by retinoids.* Methods in Enzymology, 1990. **190**: p. 100-112.

11. Silvermann, A.K., C.N. Ellis, and J.J. Voorhees, *Hypervitaminosis A syndrome; a paradigm of retinoid side effects.* Journal of the American Academy of Dermatology, 1987. **16**: p. 1027-1039.

12. Kowal, R.C., *et al., Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins.* Proceedings of the National Academy of Sciences (U.S.A.), 1989. **86**: p. 5810-5814.

13. Blomhoff, R. and T. Berg, *Isolation and cultivation of stellate cells.* Methods in Enzymology, 1990. **190**: p. 58-71.

14. Blomhoff, R. and K. Wake, *Perisinusoidal stellate cells of the liver: Important roles in retinol metabolism and fibrosis.* The FASEB Journal, 1991. **5**: p. 271-277.

15. Allen, J.G. and D.P. Bloxham, *The pharmacology and pharmacokinetics of the retinoids.* Pharmacologic Therapeutics, 1989. **40**: p. 1-27.

16. Posch, K.C., W.J. Enright, and J.L. Napoli, *Retinoic acid synthesis by cytosol from the alcohol dehydrogenase negative deermouse.* Archives of Biochemistry and Biophysics, 1989. **274**: p. 171-178.

17. Duester, G., *et al., Retinoic acid respose element in the human alcohol dehydrogenase gene ADH3: implication for regulation of retinoic acid synthesis.* Molecular and Cellular Biology, 1991. **11**: p. 1638-1646.

18. Napoli, J.L. and R.K. R., *Biogenesis of retinoic acid from β-carotene: differences between the metabolism of β-carotene and retinal.* Journal of Biological Chemistry, 1988. **263**: p. 17372-17377.

19. Giguere, V., *et al., Identification of a receptor for the morphogen retinoic acid.* Nature, 1987. **330**: p. 624-629.

20. Petkovich, M., *et al., A human retinoic acid receptor which belongs to the family of nuclear receptors.* Nature, 1987. **330**: p. 444-450.

21. Dejean, A., *et al., Hepatitis B virus DNA integration in a sequence homologous to v-erb-A and steroid receptor genes in a hepatocellular carcinoma.* Nature, 1986. **322**: p. 70-72.

22. Ishikawa, T., *et al., A functional retinoic acid receptor encoded by the gene on human chromosome 12.* Molecular Endocrinology, 1990. **4**: p. 837-844.

23. Krust, A., *et al., A third human retinoic acid receptor, hRAR-gamma.* Proceedings of the National Academy of Sciences (U.S.A.), 1989. **86**: p. 5310-5314.

24. Zelent, A., *et al., Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin.* Nature, 1989. **339**: p. 714-717.

25. Mangelsdorf, D.J., *et al., Nuclear receptor that identifies a novel retinoic acid response pathway.* Nature, 1990. **345**: p. 224-229.

26. Glass, C.K., *et al., Regulation of gene expression by retinoic acid receptors.* DNA and Cell Biology, 1991. **10**: p. 623-638.

27. Mangelsdorf, D.J., *et al., A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR.* Cell, 1991. **66**: p. 555-561.

28. Rottman, J.N., *et al., A retinoic acid-responsive element in the apolipoprotein AI gene distinguishes between two different retinoic acid response elements.* Molecular and Cellular Biology, 1991. **11**: p. 3814-3820.

29. Yu, V.C., *et al., RXRb: A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements.* Cell, 1991. **67**: p. 1251-1266.

30. Alcalay, M., *et al., Translocation breakpoint of acute promyelocytic leukemia lies within the retinoic acid receptor α locus.* Proceedings of the National Academy of Sciences (U.S.A.), 1991. **88**: p. 1977-1981.

31. Borrow, J., *et al., Molecular analysis of acute promyelocytic leukemia breakpoint cluster region of chromosome 17.* Science, 1990. **249**: p. 1577-1580.

32. de The, H., *et al., The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor a gene to a novel transcribed locus.* Nature, 1990. **347**: p. 558-561.

33. de The, H., *et al., The PML-RARα fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR.* Cell, 1991. **66**: p. 675-684.

34. Kakizuka, A., *et al., Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML.* Cell, 1991. **66**: p. 663-674.

35. Ezzell, C., *Spliced and diced receptor gene explains rare leukemia.* Journal of NIH Research, 1991. **3**: p. 55-60.

36. Breitman, T.R., S.E. Selonick, and S.J. Collins, *Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid.* Proceedings of the National Academy of Sciences (U.S.A.), 1980. **77**: p. 2936-2940.

37. Breitman, T.R., S.J. Collins, and B.R. Keene, *Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoi acid.* Blood, 1981. **57**: p. 1000-1004.

38. Nilsson, B., *Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukaemia.* British Journal of Haematology, 1984. **57**: p. 365-371.

39. Daenen, S., *et al., Retinoic acid as antileukemic therapy in a patient with acute promyelocytic leukemia and Aspergillus pneumonia.* Blood, 1986. **67**: p. 559-561.

40. Flynn, P.J., *et al., Retinoic acid treatment of acute promyelocytic leukemia: in vitro and in vivo observations.* Blood, 1983. **62**: p. 1211-1217.

41. Fontana, J.A., J.S. Rogers, and J.P. Durham, *The role of 13 cis-retinoic acid inthe remission induction of a patient with acute promyelocytic leukemia.* Cancer, 1986. **57**: p. 209-217.

42. Huang, M.E., *et al., Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia.* Blood, 1988. **72**: p. 567-572.

43. Wiernik, P.H., *Acute Leukemias,* in *Cancer: Principles and practice of oncology,* V.T.J. De Vita, S. Hellman, andS.A. Rosenberg, Editor. 1989, Lippincott: New York. p. 1809-1835.

44. Castaigne, S., *et al., All-trans retinoic acid as a differentiation therapy for acute promyeloctyic leukemia. I. Clinical results.* Blood, 1990. **76**: p. 1704-1709.

45. Chomienne, C., *et al., All-trans retinoic acid in acute promyelocytic leukemias. II. In vitro studies: Structure-function relationship.* Blood, 1990. **76**: p. 1710-1717.

46. Fenaux, P., *et al., All transretinoic acid (ATRA) in newly diagnosed acute promyeloctyic leukemia (APL): A pilot study.* Blood, 1991. **78 (Suppl. 1)**: p. 79a.

47. Chen, Z.X., *et al., A clinical and experimental study on all-trans retinoic acid-treated acute promyelocytic leukemia patients.* Blood, 1991. **78**: p. 1413-1419.

48. Warrell, R.P.J., *et al., Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid).* New England Journal of Medicine, 1991. **324**: p. 1385-1393.

49. Warrell, R.P.J., *et al., Continuous treatment with all trans-retinoic acid progressively decreases plasma drug concentrations: Implications for relapse and resistance in acute promyelocytic leukemia.* Blood, 1991. **78 (Suppl. 1)**: p. 268a.

50. Lippman, S.M., J.F. Kessler, and F.L.J. Meyskens, *Retinoids as preventive and therapeutic anticancer agents (part II).* Cancer Treatment Reports, 1987. **71**: p. 493-515.

51. Halter, S.A., *Vitamin A: Its role in the chemoprevention and chemotherapy of cancer.* Human Pathology, 1989. **20**: p. 205-209.

52. Moon, R.C. and R. Mehta, *Cancer chemoprevetion by retinoids: animal models.* Methods in Enzymology, 1990. **190**: p. 395-406.

53. Kraemer, K.H., *et al., Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin.* New England Journal of Medicine, 1988. **318**: p. 1633-1637.

54. Hong, W.K., J. Endicott, and L.M. Itri, *13-cis-retinoic acid in the treatment of oral leukoplakia.* New England Journal of Medicine, 1986. **315**: p. 1501-1505.

55. Hong, W.K., *et al., Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck.* New England Journal of Medicine, 1990. **323**: p. 795-801.

56. Willett, W.C., *Vitamin A and lung cancer.* Nutrition Reviews, 1990. **48**: p. 201-211.

57. Greenberg, E.R., et. al., *A clinical trial of beta carotene to prevent basal-cell and squamous cell cancers of the skin.* New England Journal of Medicine, 1990. **323**: p. 789-795.

58. Hennekens, C.H. and K. Eberlein, *A randomized trial of aspirin and β–carotene among US physicians.* Preventive Medicine, 1985. **14**: p. 165-168.