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Retinoids

The Biochemical and Molecular Basis of Vitamin A and Retinoid Action

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CHAPTER 10

The Retinoids: Cancer Therapy and Prevention Mechanisms

K. NASON-BURCHENAL and E. DMITROVSKY

I. Summary

The retinoids, synthetic and natural derivatives of vitamin A, are ligands activating nuclear transcription factors that are members of the steroid receptor superfamily. Ligand-retinoid receptor interactions lead to activation or repression of target genes which in turn signal retinoid biological actions. There is a convergence of basic scientific and clinical findings in the retinoid field. As a fuller understanding of the retinoid signaling pathway has evolved, beneficial clinical effects were found using the retinoids in cancer therapy and prevention. Underscoring this convergence is the tight link that often exists between observed clinical retinoid responses and activation or repression of specific retinoid receptors. This chapter discusses this convergence through an analysis of retinoid-based cancer therapy and prevention mechanisms. Attention focuses on how an understanding of the retinoid signaling pathway provides insight into mechanisms responsible for retinoid clinical responses.

II. Retinoid Clinical Activities

Vitamin A is essential for vision and for fertility. It plays an important role in development and in regulation of the growth and maturation states of normal and neoplastic cells. Vitamin A also exerts major effects on immune function. In this chapter, attention is placed on the retinoid role in cancer therapy and prevention. While the teratogenic effects of the retinoids are recognized (GUDAS et al. 1994; ROTHMAN et al. 1995) and limit their use in women of child-bearing years, discussion of these biological effects are beyond the scope of this chapter. The therapeutic role of the retinoids in nonneoplastic diseases is also outside the scope of this chapter.

The retinoids have reported clinical activity in cancer therapy and prevention. Clinical activity is reported in several settings. These include treatment of the premalignant lesions oral leukoplakia (HONG et al. 1986; LIPPMAN et al. 1993), actinic keratosis (LIPPMAN et al. 1987), cervical dysplasia (MEYSKENS et al. 1994), and xeroderma pigmentosum (KRAEMER et al. 1988). Reduction in second hepatocellular (MUTO et al. 1996) or aerodigestive tract cancers (HONG et al. 1990; PASTORINO et al. 1993) follows retinoid treatments. Some established malignancies respond to retinoid-based therapy and include:

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acute promyelocytic leukemia (APL) (HUANG et al. 1988; CASTAIGNE et al. 1990; WARRELL et al. 1991), juvenile chronic myelogenous leukemia (CASTLEBERRY et al. 1994), and mycosis fungoides (WARRELL et al. 1983). In combination with interferon- α 2a, 13-*cis*-retinoic acid (13-*cis*-RA) is reported as active in treating squamous cell cancers of the skin (LIPPMAN et al. 1992a) and cervix (LIPPMAN et al. 1992b) or disseminated kidney cancer (MOTZER et al. 1995). Nutritional intervention trials in Linxian, China with vitamin and mineral combinations have also affected cancer incidence and disease specific mortality (BLOT et al. 1993). The following is a summary of the retinoid clinical activity in cancer therapy and prevention:

Treatment of premalignant lesions

- Oral leukoplakia
- Cervical dysplasia
- Xeroderma pigmentosum
- Actinic keratosis

Chemoprevention

- Second lung cancers
- Second head and neck cancers
- Second hepatocellular carcinomas

Treatment of overt malignancies

- Acute promyelocytic leukemia
- Juvenile chronic myelogenous leukemia
- Mycosis fungoides

Combination therapy

- Squamous cell skin cancer
- Squamous cell cervical cancer
- Kidney cancer

The observed reduction in second malignancies of the aerodigestive tract or liver following retinoid treatments raised the prospect of retinoid-based prevention of cancers in high-risk individuals. To begin to test this possibility, patients who were at risk for aerodigestive tract cancers due to tobacco use were treated with carotenoid-based prevention regimens. Unfortunately, clinical cancer prevention activity was not observed in this setting with the tested carotenoid (ALPHA TOCOPHEOL, BETA CAROTENE CANCER PREVENTION STUDY GROUP 1994; OMENN et al. 1996; HENNEKENS et al. 1996). The combination of beta carotene and vitamin A was also found not to reduce lung cancer incidence (OMENN et al. 1996). Given the high cost and long duration of such clinical prevention trials, alternative approaches need to be developed to assess which of the retinoids exhibit cancer prevention activity. This chapter summarizes the present knowledge about the retinoid signaling pathway to understand how retinoids act in cancer therapy and prevention. Key elements of the retinoid signaling pathway are identified, as reviewed in Fig. 1. The way in

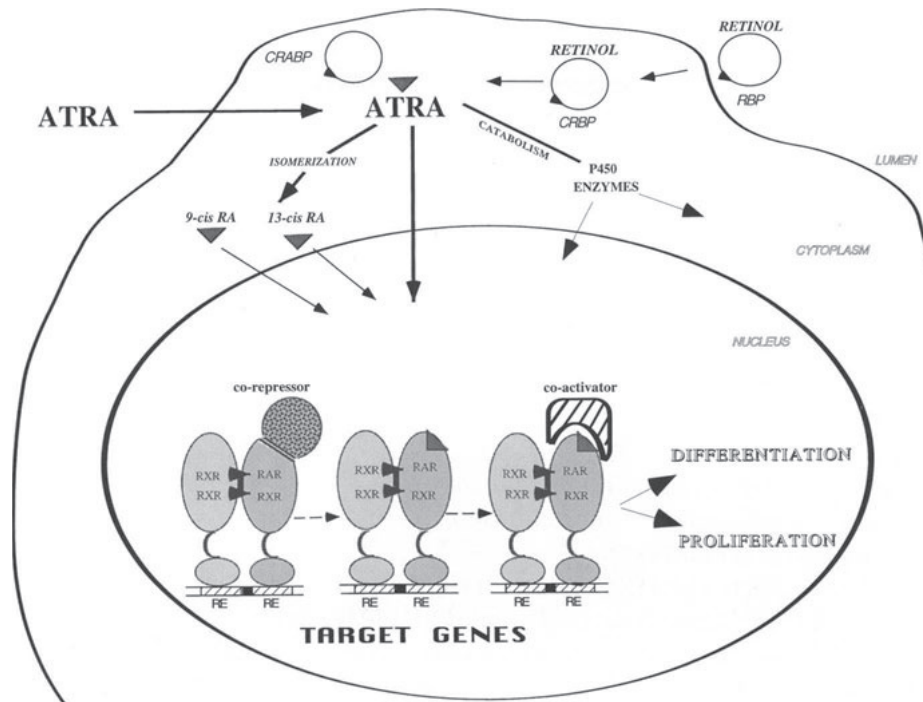


Fig. 1. The retinoid signaling pathway. This figure depicts the key components involved in all-*trans*-retinoic acid (ATRA) signaling. These components include the retinol-binding protein (RBP) present in the lumen, the cytosolic retinoid binding proteins (CRABP and CRBP), catabolic and modifying enzymes present in the cytoplasm, and the nuclear retinoid receptors (RARs and RXRs) which interact with defined DNA response elements (RE). Also depicted are important protein-protein interactions found between retinoid receptors and coactivators or corepressors that contribute to the ligand-dependent activation of target genes which directly transmit retinoid growth and differentiation signals. *Closed triangles*, ligands. Two stereoisomers of ATRA are depicted, including 9-*cis*-retinoic acid (9-*cis*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA). (copyright by the American Society of Clinical Oncology Educational Book)

which these components play a critical role in clinical retinoid responses is discussed.

III. Mechanisms of Retinoid Action

Important components of the retinoid signaling pathway include cytoplasmic and nuclear retinoid receptors. Cytosolic and nuclear receptors are isolated and sequenced. These are structurally and functionally distinct receptors. Cytosolic retinoid binding proteins are identified (STONER and GUDAS 1989; GIGUÈRE et al. 1990). These cytosolic receptors can bind retinoids but not DNA. Their precise functions in the retinoid signaling pathway are being

defined. These receptors may serve roles as intracellular retinoid storage sites.

The nuclear retinoid receptors are members of the steroid receptor superfamily, including vitamin D receptor (McDONNEL et al. 1987), estrogen receptor (GREEN and KUMAR 1987), and thyroid hormone receptors (WEINBERGER et al. 1986), among others. These are hormone dependent transcription factors. The retinoid nuclear receptors bind specific retinoids through ligand binding domains and interact with DNA through DNA binding domains. These DNA-receptor and ligand-receptor interactions transcriptionally activate or repress target genes that mediate retinoid growth and differentiation effects. There are two families of nuclear retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Three RARs exist with multiple isoforms: RAR α (PETKOVICH et al. 1987; GIGUÈRE et al. 1987), RAR β (BRAND et al. 1988), and RAR γ (KRUST et al. 1989). RAR α is located on the long arm of chromosome 17 (MATTEI et al. 1988) at the site of the t(15;17) acute promyelocytic leukemia breakpoint. The chromosomal locations of the other RARs are known (MATTEI et al. 1991).

Three RXRs exist: RXR α (MANGELSDORF et al. 1990), RXR β (HAMADA et al. 1989; MANGELSDORF et al. 1992), and RXR γ (MANGELSDORF et al. 1992; LEID et al. 1992). These retinoid receptors were known as orphan receptors until a physiological ligand was found: 9-*cis*-retinoic acid (9-*cis*-RA) (HEYMAN et al. 1992; LEVIN et al. 1992). The RXRs heterodimerize with other steroid receptor superfamily members, as reported (HAMADA et al. 1989; MANGELSDORF et al. 1992; LEID et al. 1992; HEYMAN et al. 1992; LEVIN et al. 1992; MANGELSDORF et al. 1991; KLIEWER et al. 1992; ZHANG et al. 1992a). Heterodimerization plays a role in cooperation between receptor-dependent signaling pathways. Different ligands are reported to favor homodimer versus heterodimer formation. For instance, 9-*cis*-RA favors formation of RXR-RXR homodimers versus RAR-RXR heterodimers (ZHANG et al. 1992b). The biological effects of these distinct dimerization patterns may depend on the cell context examined. Understanding the function of specific retinoid receptors in signaling the biological effects of the retinoids is assisted by development of agonists and antagonists for specific retinoid receptors which have been studied during induced tumor cell differentiation (LEHMANN et al. 1991, 1992; CHIBA et al. 1997). It is now recognized protein-protein interactions with coregulators have major effects on signaling retinoid receptor actions through associations with stimulatory coactivators or inhibitory corepressors that interact with the basal transcriptional machinery (CHEN and EVANS 1995; CHAKRAVARTI et al. 1996; KUROKAWA et al. 1995; OÑATE et al. 1995; GLASS et al. 1997, and references therein).

While synthetic retinoids are engineered to activate or inhibit transcription of specific retinoid receptors (LEHMANN et al. 1991, 1992), others exist that antagonize transcription of AP-1 (FANJUL et al. 1994). Some retinoids signal effects through receptor independent mechanisms. One example is N-(4-hydroxyphenyl)retinamide (4HPR). Although able to transcriptionally acti-

vate $RAR\gamma$ (FANJUL et al. 1996), 4HPR is viewed as predominantly signaling effects through receptor independent mechanisms (DELIA et al. 1993, 1997; SHEIKH et al. 1995; ORIDATE et al. 1996, 1997). 4HPR preferentially signals apoptosis even in retinoid-resistant cells (DELIA et al. 1993). That 4HPR triggers apoptosis through novel mechanisms is supported by the finding its biological effects are signaled through induction of reactive oxygen species in some cell contexts (DELIA et al. 1997; ORIDATE et al. 1997). Another retinoid, designated AHPN, also appears to signal through retinoid receptor independent mechanisms (SHAO et al. 1995).

A tight link exists between expression of specific retinoid receptors and preclinical or clinical retinoid responses, as summarized below. Preclinical or clinical findings are displayed that indicate a link between retinoid biological responses and expression of specific retinoid receptors. Examples of a link between expression of specific retinoid receptors and beneficial clinical responses are found in oral leukoplakia where basal $RAR\beta$ expression is repressed and 13-*cis*-retinoic acid induces $RAR\beta$ expression in responding lesions. In acute promyelocytic leukemia, expression in leukemic cells of the t(15;17) rearranged receptor, PML/ $RAR\alpha$, identifies those patients responsive to all-*trans*-retinoic acid therapy (ATRA) (in some cases an association between expression of specific receptors and clinical or preclinical responses has not yet been reported, indicated below by “–”):

- Oral leukoplakia: $RAR\beta$
- Squamous cell lung cancer: $RAR\beta$
- Transformed bronchial epithelium: $RAR\alpha + RAR\beta$
- Acute promyelocytic leukemia: PML/ $RAR\alpha$
- Myeloid leukemia (HL-60): $RAR\alpha$
- Kidney cancer: $RAR\beta$
- Breast cancer: $RAR\alpha + RAR\beta$
- Male germ cell cancer: $RAR\gamma$
- Cervical cancer: –
- Squamous cell skin cancer: –
- Mycosis fungoides: –
- Juvenile chronic myelogenous leukemia: –
- Aerodigestive tract chemoprevention: –
- Hepatocellular carcinoma chemoprevention: –

One example is illustrated in the premalignant lesion oral leukoplakia. In this example, an association between retinoid-induced $RAR\beta$ mRNA expression and favorable clinical response to 13-*cis*-RA treatment is reported (LOTAN et al. 1995). Another example is acute promyelocytic leukemia where expression of the fusion product of the t(15;17) rearrangement, PML/ $RAR\alpha$, predicts clinical response to (ATRA)-treatment (MILLER et al. 1992). Other examples of a link between preclinical retinoid responses and expression of specific retinoid receptors are presented above. These include results of transfection

experiments revealing $RAR\alpha$ mediates retinoid maturation response in myeloid leukemia (COLLINS et al. 1990), $RAR\beta$ triggers retinoid growth suppressive response in squamous cell lung carcinoma cells (HOULE et al. 1993) and $RAR\gamma$ mediates retinoid growth and differentiation responses in human embryonal carcinomas (MOASSER et al. 1994, 1995b; SPINELLA et al. 1998). In transformed human bronchial epithelial cells, $RAR\alpha$ and $RAR\beta$ are mediators of retinoid-dependent growth suppression (AHN et al. 1995a). These findings and others obtained from transfection experiments provide a rationale for use of retinoid-receptor selective agonists to treat defined neoplastic diseases.

IV. Retinoids and Cancer Chemoprevention

A major therapeutic potential role for the retinoids is found in cancer prevention. The concept of cancer chemoprevention, first coined by SPORN et al. (1976), formulated the therapeutic rationale for treatments at early stages of carcinogenesis, before overt malignancies develop. Chemoprevention strategies can be directed towards the initiation, promotion, or progression stages of carcinogenesis. Several carcinogenesis stages might be targeted, including invasive or metastatic stages. If treatment strategies are successful in reversing or preventing progression of early neoplastic lesions, the major clinical consequences of malignancies would be reduced.

Based on preclinical findings (WOLBACH and HOWE 1925), epidemiological evidence (reviewed in HONG et al. 1994), data derived from experimental animal models (MOON et al. 1994), and successful clinical trials treating premalignant lesions or preventing second malignancies (HONG et al. 1986; LIPPMAN et al. 1993; LIPPMAN et al. 1987; KRAEMER et al. 1988; MUTO et al. 1996; HONG et al. 1990; PASTORINO et al. 1993), there is a strong rationale for use of retinoids in cancer chemoprevention. As reported by WOLBACH and HOWE in 1925, vitamin A is essential for epithelial cell homeostasis. Studies with vitamin A deficient rodents indicate squamous metaplasia develops. These metaplastic lesions are reminiscent of those detected in active smokers. Notably, when this vitamin A deficiency is repleted, the squamous metaplasia is reversed. This finding, when coupled with the epidemiological evidence of an inverse relationship between site-specific cancer incidences and serum vitamin A or β -carotene levels (reviewed in HONG et al. 1994), provides a strong rationale to conduct retinoid-based cancer prevention clinical trials. That β -carotene appears inactive in prevention of aerodigestive tract tumors in studied patients (ALPHA TOCOPHEROL, BETA CAROTENE CANCER PREVENTION STUDY GROUP 1994; OMENN et al. 1996; HENNEKENS et al. 1996) underscores the need to have an earlier assessment of chemoprevention response than is currently available when clinical outcome is the only end point used to assess the potential therapeutic impact. If clinical outcome is the sole outcome used to evaluate response, progress in retinoid-based cancer prevention will not likely be rapid.

Reliable “intermediate markers” are needed as surrogate endpoints for chemoprevention trials. Ideally, these would reflect early changes in affected epithelium useful to monitor effective clinical chemoprevention responses. Intermediate markers may identify individuals at high-risk for malignant transformation of precursor lesions. Alternatively, absence of such epithelial changes would identify those lesions least likely to develop into overt malignancies. In 1953 SLAUGHTER and colleagues proposed the existence of carcinogen-exposed epithelial “fields” of altered epithelial cells preceding development of invasive malignancies. The field cancerization hypothesis and the concept of multistep carcinogenesis are established and well defined especially in colon carcinogenesis (FEARON and VOGELSTEIN 1990). While these concepts have guided thinking in clinical cancer prevention, it is perhaps surprising that it remains to be seen which genetic changes are rate-limiting for maintenance or progression of premalignant lesions. These rate-limiting changes may differ for each carcinogenic program. In support of this view, frequent and distinct genetic changes are reported in diverse epithelial sites including the colon (FEARON and VOGELSTEIN 1990), head and neck (CALIFANO et al. 1996), lung (HUNG et al. 1995; RUSCH et al. 1995), and others. Which pathways are essential for development or maintenance of the transformed phenotype need to be determined since these are attractive therapeutic targets for cancer prevention.

Some changes associated with epithelial cell transformation are altered by effective retinoid chemoprevention treatments. Other changes predict clinical retinoid resistance. One example of an association between expression of a specific retinoid receptor and beneficial retinoid clinical response is found in the repressed expression of $RAR\beta$ present in premalignant aerodigestive tract lesions, including oral leukoplakia (LOTAN et al. 1995). After 13-*cis*-RA treatment of patients with oral leukoplakia, $RAR\beta$ mRNA expression is induced in clinically responsive lesions (LOTAN et al. 1995). In contrast, aberrant p53 protein expression in oral leukoplakia lesions predicts clinical retinoid resistance (LIPPMAN et al. 1995). While mechanisms responsible for retinoid resistance triggered by aberrant p53 expression are not yet identified, the finding of clinical retinoid resistance occurring in oral leukoplakia lesions overexpressing p53 illustrates how genetic or immunohistochemical analyses of premalignant epithelial lesions can reveal those lesions likely to progress to an irreversibly transformed phenotype or those responsive to retinoid-based chemoprevention (LIPPMAN et al. 1995). As microarray technologies advance, perhaps those expressed mRNA species indicative of the transformed phenotype will be determined in patient-derived specimens.

One useful strategy to reveal chemoprevention mechanisms is to exploit clinical responses to guide basic scientific studies. While retinoids are reported to reduce second malignancies of the head and neck (HONG et al. 1990), lung (PASTORINO et al. 1993), or liver (MUTO et al. 1996), carotenoid-based cancer chemoprevention of aerodigestive tract tumors in high risk individuals has not been SUCCESSFUL (ALPHA TOCOPHEROL, BETA CAROTENE CANCER PREVENTION

STUDY GROUP 1994; OMENN et al. 1996; HENNEKENS et al. 1996). Perhaps differences in activated or repressed target genes triggered by retinoid but not carotenoid treatments account for these observed differences in clinical responses. Analysis of mechanisms responsible for these differences should identify pathways linked to effective cancer chemoprevention. Another approach taken in pursuit of retinoid-based cancer therapy or prevention mechanisms is to study relevant in vitro models.

V. In Vitro Models for Retinoid Activity in Cancer Therapy and Prevention

To understand how retinoids act in cancer therapy and prevention, in vitro models reflective of clinical retinoid activities are available for study. Hematopoietic and nonhematopoietic models exist and are useful to reveal retinoid-dependent growth and differentiation signals. Several in vitro models are often used to evaluate retinoid growth and differentiation signals in human tumors. These include: the multipotent human embryonal carcinoma cell line NTERA-2 clone D1 (abbreviated NT2/D1), the APL cell line NB4, and the immortalized human bronchial epithelial cell line BEAS-2B, among others. The distinct features of these models are discussed below.

A. The Multipotent NT2/D1 Human Embryonal Carcinoma Line

The NT2/D1 human embryonal carcinoma cell line is multipotent, differentiating into a neuronal phenotype and other lineages after ATRA-treatment (ANDREWS 1984; ANDREWS et al. 1984). ATRA-treatment represses NT2/D1 tumorigenicity (DMITROVSKY et al. 1990a). Hexamethylene bisacetamide (HMBA) induces a nonneuronal phenotype (MILLER et al. 1994) with repressed tumorigenic potential. The undifferentiated NT2/D1 cells express the stage specific embryonal antigen, SSEA-3 (ANDREWS et al. 1990; FENDERSON et al. 1987). Following ATRA or HMBA treatments, SSEA-3 marker expression declines (MILLER et al. 1994). The neuronal antigen recognized by the A2B5 monoclonal antibody is induced by ATRA-treatment (MILLER et al. 1994; FENDERSON et al. 1987) but not by HMBA-treatment (MILLER et al. 1994). This in vitro NT2/D1 differentiation model is clinically relevant since maturation is common in clinical germ cell tumors. This is reflected in the frequent appearance of mature teratoma in some clinical germ cell tumors (HONG et al. 1977).

When compared to F9 murine teratocarcinomas, the human NT2/D1 line has distinct features. These include: (a) requirement for high ATRA doses to efficiently signal terminal differentiation (KURIE et al. 1993b); (b) a multipotent response to ATRA (ANDREWS 1984; ANDREWS et al. 1984); (c) distinct phenotypes induced following combined treatments with dibutyryl cAMP and ATRA or combined treatments with phorbol ester and ATRA (KURIE et al.

1993a,c, 1994); (d) unique immunophenotypic maturation markers (FENDERSON et al. 1987); and (e) a marker chromosomal abnormality in NT2/D1 and other human germ cell tumors, an isochromosome of 12p (DMITROVSKY et al. 1990b; BOSL et al. 1989; MURTY et al. 1990).

Findings obtained using the NT2/D1 embryonal carcinoma cell line are more reflective of the differentiation potential of human germ cell tumors than studies performed using available murine teratocarcinoma models. To determine which retinoid receptor is responsible for terminal differentiation of these human embryonal carcinoma cells, retinoid-resistant NT2/D1 cells were derived via mutagenization and selection in increasing ATRA doses. Notably, all ATRA-resistant clones derived and a spontaneously retinoid resistant embryonal carcinoma cell line, N2102Ep, aberrantly expressed RAR γ (MOASSER et al. 1996). Over-expression of wild-type RAR γ in a retinoid resistant NT2/D1 clone, designated NT2/D1-R1 and having altered RAR γ expression, reversed retinoid resistance but did not restore the complete retinoid differentiation response (MOASSER et al. 1994, 1995b). Over-expression of RAR γ in parental NT2/D1 cells leads to terminal growth suppression and induction of a mesenchymal phenotype, not a neuronal phenotype (MOASSER et al. 1995b).

In search for other retinoid receptors which might trigger terminal differentiation in these human embryonal carcinoma cells, each of the RARs and the RXRs were individually transfected into parental NT2/D1 cells. Results reveal of the RARs only RAR γ induces terminal NT2/D1 morphological maturation and growth suppression (MOASSER et al. 1995b; SPINELLA et al. 1998). Of the RXRs, only RXR β triggers in these cells morphological maturation and terminal growth suppression (SPINELLA et al. 1998). The phenotypes induced by ATRA-treatment versus RAR γ or RXR β transfection are distinct. To determine whether cooperation between RAR and RXR pathways are required to elicit the full retinoid response, RAR γ and RXR β were cotransfected into NT2/D1 cells. This yielded a neuronal phenotype reminiscent of that following RA-treatment (SPINELLA et al. 1998). Thus, these findings indicate cooperation between RAR and RXR pathways is required to trigger the full retinoid response in human embryonal carcinoma cells. Perhaps an analogous cooperation is active in other cell contexts.

Whether retinoid-treatment of chemotherapy-resistant clinical germ cell cancers signals tumor cell differentiation or antitumor responses was studied using receptor nonselective retinoids including 13-*cis*-RA and ATRA (GOLD et al. 1984; MOASSER et al. 1995a). While extensive teratoma formation was identified in some chemotherapy-resistant germ cell cancer patients treated with 13-*cis*-RA and stable disease was noted in others, objective antitumor responses were not reported following treatment with 13-*cis*-RA or ATRA (GOLD et al. 1984; MOASSER et al. 1995a). Whether retinoid receptor selective agonists that activate RAR γ or RXR β trigger major antitumor responses through germ cell tumor differentiation is the subject of future clinical work.

B. Acute Promyelocytic Leukemia In Vitro Models

ATRA-treatment of APL cases that contain the t(15;17) rearrangement induces leukemic cell maturation and transient complete remissions (HUANG et al. 1988; CASTAIGNE et al. 1990; WARRELL et al. 1991). Paradoxically, these clinical ATRA responses are associated with expression of the t(15;17) fusion product, PML/RAR α (MILLER et al. 1992). To understand how ATRA-treatment triggers differentiation in APL, the NB4 APL cell line (LANOTTE et al. 1991) has proven useful to study. This is an APL line containing the t(15;17) rearrangement and expressing PML/RAR α . Variant translocations also exist and involve RAR α rearrangements (CHEN et al. 1993; REDNER et al. 1996; WELLS et al. 1997). In NB4 APL cells and in clinical APL cases, the PML/RAR α fusion transcript is expressed in leukemic cells with the known or putative transcription factors: PML and RAR α . These are the products of the nonrearranged alleles from chromosomes 15 and 17, respectively. Findings indicate PML/RAR α is a ligand-dependent transcription factor with dominant-negative functions exerted on PML, RAR α , both PML and RAR α , or down-stream pathways (DE THÉ et al. 1991; KAKIZUKA et al. 1991; KASTNER et al. 1992). Transfection experiments conducted in the leukemic cell lines, U937, K562, or HL-60, which lack PML/RAR α expression, indicate inhibitory effects of PML/RAR α (ROUSSELOT et al. 1994; GRIGNANI et al. 1993, 1995) on these leukemic cells.

In addition to inhibitory effects on leukemic cell maturation by engineered PML/RAR α expression, inhibitory effects on the PML pathway are reported. Within t(15;17) APL cells, PML is associated with nuclear bodies, designated as PML oncogenic domains (PODs). ATRA-treatment reverses this abnormal POD distribution pattern (DYCK et al. 1994; KOKEN et al. 1994; WEISS et al. 1994). Antagonism of RAR α -dependent transcription also occurs when PML/RAR α is cotransfected in transient transfection assays (DE THÉ et al. 1991; KAKIZUKA et al. 1991; KASTNER et al. 1992). That PML/RAR α triggers leukemogenesis is shown through transgenic lines evaluating PML/RAR α expression in myeloid cells. While all reported lines develop impairment of myelopoiesis, in some lines leukemia occurs after a long latency in a subset of mice (EARLY et al. 1996; GRISOLANO et al. 1997; BROWN et al. 1997; HE et al. 1997). That a long latency is required for the development of overt leukemia suggests a second hit is needed to trigger leukemogenesis. The nature of this additional oncogenic event is not yet known.

The NB4 line is useful to evaluate mechanisms of retinoic acid response in an APL cell line that reflects key aspects of the biology of this leukemia and of clinical retinoid response (LANOTTE et al. 1991). As discussed, this line contains the t(15;17) balanced rearrangement and expresses PML/RAR α (LANOTTE et al. 1991; KAKIZUKA et al. 1991). Before ATRA-treatment, NB4 cells have an immature myeloid phenotype and are highly proliferative and tumorigenic. After ATRA-treatment, a mature myeloid phenotype is induced with expression of the epitope recognized by the myelomonocytic immuno-

phenotypic marker, MO-1. These differentiated cells have acquired ability to reduce nitroterazolium blue, a marker of mature myeloid function. These ATRA-differentiated NB4 cells have a more limited growth potential than undifferentiated NB4 cells. These differentiated cells have negligible clonal growth potential or tumorigenic capacity (LANOTTE et al. 1991; NASON-BURCHENAL et al. 1997; K. N.-B., unpublished observations). Thus, this line reflects key aspects of the ATRA differentiation response observed in APL cells of ATRA-treated t(15;17) patients.

To comprehensively evaluate the PML/RAR α role in ATRA-signaling in the NB4 APL cell line, the growth and differentiation effects of transcription factors involved in retinoid signaling of differentiation have been evaluated. Using gain of function experiments, the products of the nonrearranged alleles, RAR α and PML were each over-expressed in NB4 cells (AHN et al. 1995b; MU et al. 1994). When RAR α or PML are individually over-expressed in NB4 cells, growth suppression is signaled. For RAR α over-expression, maturation response is enhanced (AHN et al. 1995b). These findings indicate reducing PML/RAR α levels relative to the nonrearranged alleles, RAR α or PML, antagonizes the inhibitory effects of PML/RAR α . Building on this view is the finding that engineered over-expression of RXR α in NB4 cells causes marked growth suppression of transfectants (NASON-BURCHENAL et al. 1997). Since RXR is a known heterodimerization partner of PML/RAR α , these findings suggest sequestration of PML/RAR α antagonizes the leukemogenic effects of PML/RAR α . These gain of function studies, when coupled with the findings obtained using transgenic mice (EARLY et al. 1996; GRISOLANO et al. 1997; BROWN et al. 1997; HE et al. 1997), indicate the inhibitory effect of PML/RAR α is overcome by ATRA-treatment.

To complement these studies, loss of function experiments were conducted using molecular genetic strategies to target PML/RAR α expression. Two approaches taken include: homologous recombination at the PML/RAR α locus in NB4 cells and hammerhead ribozymes engineered to inactivate PML/RAR α mRNA in APL cells (PACE et al. 1994; NASON-BURCHENAL et al. 1998a,b). PML/RAR α targeting experiments indicate persistent PML/RAR α expression is required for maintenance of basal promyelocytic leukemic cell growth. This is shown to be the case for retinoid sensitive NB4 cells and retinoid resistant NB4 cells that harbor a mutation within the ligand binding domain of the RAR α portion of PML/RAR α (NASON-BURCHENAL et al. 1998a,b). Targeting of PML/RAR α yields a lethal phenotype associated with prominent induction of apoptosis (NASON-BURCHENAL et al. 1998a,b). Taken together, these loss of function experiments (NASON-BURCHENAL et al. 1998a,b) and gain of function experiments using engineered PML/RAR α expression in myeloid leukemic cells (ROUSSELOT et al. 1994; GRIGNANI et al. 1993, 1995), indicate PML/RAR α acts as an antiapoptotic transcription factor. ATRA-treatment overcomes these antiapoptotic effects. While a full explanation for the clinical retinoid response in t(15;17) APL cases does not yet exist, the existence of cellular and transgenic models and useful molecular genetic tools such

as hammerhead ribozymes to target PML/RAR α expression should provide additional mechanistic insight into APL biology. Application of these reagents could lead to a resolution of the apparent paradoxical response to ATRA-treatment in APL cases. In addition to the PML/RAR α role in leukemogenesis, PML/RAR α cofactors and the histone deacetylase complex appear to play important roles in retinoid response (LIN et al. 1998; GRIGNANI et al. 1998). The existence of PML “knock-out” mice provides an opportunity to learn how PML or its target genes contribute to leukemogenesis (WANG et al. 1998).

Another approach to understand important mechanisms responsible for ATRA-maturation response in APL is to derive ATRA-resistant APL cells and to explore acquired defects accounting for retinoid resistance. Several groups reported ATRA-resistant NB4 APL lines (NASON-BURCHENAL et al. 1997; DUPREZ et al. 1992; DERMINE et al. 1993; SHAO et al. 1997; RUCHAUD et al. 1994). Common defects of different retinoic acid resistant promyelocytic leukemia cells include persistent telomerase activity and nuclear body disorganization (NASON-BURCHENAL et al. 1997). In some ATRA-resistant APL lines, mutations within the ligand binding domain of the RAR α portion of PML/RAR α are found (NASON-BURCHENAL et al. 1998a; SHAO et al. 1997). This suggests a dominant-negative PML/RAR α contributes to acquired retinoid resistance. Alternatively, a role for second messenger pathways are implicated in overcoming retinoid resistance (RUCHAUD et al. 1994). That persistent expression of an altered PML/RAR α is required for maintenance of leukemic cell growth is shown through targeting of the PML/RAR α transcript derived from an NB4 retinoid resistant line that contains a ligand binding domain mutation in the RAR α portion of PML/RAR α (NASON-BURCHENAL et al. 1998a).

During the ATRA-treatment of t(15;17) APL patients, the majority of treated patients achieve clinical remissions. Yet, retinoid resistance occurs following single-agent ATRA therapy. Several hypotheses are offered to account for this retinoid resistance including induction of retinoic acid-binding proteins (CORNIC et al. 1992) and pharmacological mechanisms of resistance (MUINDI et al. 1992). These clinical mechanisms of APL ATRA-resistance need not be mutually exclusive. Perhaps those mechanisms shown as active in the in vitro setting of ATRA-resistant NB4 cells will be active in vivo in APL patients resistant to ATRA-based therapy. Whether mutations are frequently found in the ligand binding domain of the RAR α portion of PML/RAR α of APL cells derived from patients exhibiting retinoid resistance needs to be learnt. Future work will determine whether ATRA-resistant cell lines are useful to pursue clinically relevant mechanisms to overcome retinoid resistance in APL patients.

While ATRA-treatment induces remissions in t(15;17) APL cases, the PLZF-RAR α variant translocation cases appear retinoid resistant (CHEN et al. 1993). The clinical retinoid responses of APL variant translocation cases need to be studied in greater depth. For t(15;17) APL cases, ATRA-treatment combined with chemotherapy induces long-term disease-free survival superior

to that achieved with combination chemotherapy alone (DEGOS et al. 1995; FENAUX et al. 1993; FRANKEL et al. 1994; TALLMAN et al. 1997). The optimal scheduling and doses used for combining ATRA with chemotherapy regimens need to be determined.

C. An In Vitro Lung Cancer Prevention Model

Given the long duration and large size of clinical cancer chemoprevention trials using retinoids or other prevention agents, there is a need for in vitro models predictive of clinical cancer prevention activities. While reduction in second malignancies is reported for aerodigestive tract and hepatocellular cancers (MUTO et al. 1996; HONG et al. 1990; PASTORINO et al. 1993), the optimal retinoids active in each of these clinical settings have not yet been identified. This view is supported by clinical trials showing β -carotene treatment is ineffective in preventing lung cancers from arising in high risk individuals (ALPHA TOCOPHEROL, BETA CAROTENE CANCER PREVENTION STUDY GROUP 1994; OMENN et al. 1996; HENNEKENS et al. 1996).

To determine which retinoids are most active in lung cancer prevention, an in vitro model (REDDEL et al. 1988) was adapted to study how carcinogens mediate malignant transformation of immortalized human bronchial epithelial cells. This model uses T antigen-immortalized human bronchial epithelial cells and exposure of these cells to tobacco-derived carcinogens to assess whether retinoids antagonize transformation and to reveal pathways responsible for effective in vitro prevention (LANGENFELD et al. 1996). This model has revealed that ATRA inhibits malignant transformation of these epithelial cells by carcinogens (LANGENFELD et al. 1996). This in vitro cancer prevention activity is linked to a delay in the G₁-S cell cycle transition signaled through a decline in expression of cyclin E (LANGENFELD et al. 1996) and cyclin D (LANGENFELD et al. 1997) proteins. This ATRA-mediated decline in cyclin expression is signaled through posttranslational mechanisms activating cyclin proteolysis via a proteasome-dependent degradation pathway (LANGENFELD et al. 1997).

This model appears useful to identify transformation pathways activated by carcinogen exposure. It also reveals which pathways are inhibited by effective retinoid-based prevention in the in vitro setting. Whether these transformation pathways are antagonized during effective clinical chemoprevention is not yet known. How other retinoid dependent mechanisms are engaged during clinical cancer chemoprevention is being explored. Retinoid receptor-selective agonists and antagonists exist that may have reduced pharmacological toxicities as compared to receptor nonselective retinoids. As retinoid receptor-dependent pathways are identified as active in signaling cancer prevention, these agents will become available for testing in appropriate clinical trials. Perhaps the retinoids will ultimately prove most useful as pharmacological tools to identify anticarcinogenic pathways that are more optimally targeted by other therapeutic agents. Of course, nonretinoid prevention agents exist

with potential clinical cancer chemoprevention activities. These agents affect estrogen-dependent signaling, inducible cyclooxygenase, or other pathways relevant to cancer prevention, as reviewed (HONG and SPORN 1997). Cooperation between the retinoids and other chemoprevention agents may augment clinical cancer prevention activities.

VI. Summary and Future Directions

The retinoids, synthetic and natural derivatives of vitamin A, are pharmacological agents active in cancer therapy and prevention. There is a convergence of basic and clinical findings in the retinoid field. Clinical progress in this field has occurred along with a fuller understanding of which nuclear retinoid receptors or coregulators transmit retinoid growth and differentiation signals. That some clinical responses are observed as linked to expression or induction of specific retinoid receptors indicate how receptor-selective pathways are responsible for these clinical actions. These findings, when coupled with available preclinical data, provide a strong rationale for use of retinoid receptor-selective agonists as therapeutic agents for treatment of neoplastic diseases.

One way to optimize the use of receptor selective or nonselective retinoids is to explore biological effects in *in vitro* models that reflect activity observed in clinical cancer therapy or prevention. The retinoids may eventually prove most useful to highlight key growth, differentiation, or anticarcinogenic pathways that are more favorably targeted by other agents. Whether receptor selective retinoids reduce pharmacological toxicities, as compared with receptor-nonselective retinoids, will be learned. How clinical efficacy of these retinoids are affected by their increased selectivity is not yet known. Identification of target genes that transmit retinoid biological effects is needed. These target genes might be distinct for different neoplastic settings. The pharmacological regulation of target genes could alter the aberrant growth or differentiation of neoplastic cells.

How the retinoids are clinically active in combination therapy with other ligands of the steroid-receptor superfamily, cytokines, growth factor agonists or antagonists, radiation therapy, chemotherapeutic agents, or other agents needs to be further studied. *In vitro* experiments indicate cooperation is already observed between second messenger pathways and the retinoid signaling pathway (KURIE et al. 1993a,c, 1994; RUCHAUD et al. 1994). Clinical cooperation between the retinoid and interferon signaling pathways is established in the treatment of squamous cell cancers of the skin, cervix, and in disseminated kidney cancer (LIPPMAN et al. 1992a,b; MOTZER et al. 1995). How best to use retinoids as single agents or as part of combination therapy regimens is under study. The clinical challenge is to understand how the retinoids act and when these agents should be used in the clinic. Pursuit of basic mechanisms responsible for retinoid biological effects will optimize their use in cancer therapy and prevention.

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