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Recent Advances in the Use of Vitamin A (Retinoids) in the Prevention and Treatment of Cancer

Running Title: **Vitamin A and Cancer**

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ABSTRACT

Vitamin A, its physiological metabolites and synthetic derivatives (retinoids) have been shown to have protective effects against the development of certain types of cancer. In addition, pharmacological amounts of retinoids have been used with some success in the treatment of a few human tumors. The chemoprevention effect of retinoids is most likely exerted at the tumor promotion phase of carcinogenesis. Retinoids block tumor promotion by either inhibiting proliferation, inducing apoptosis, inducing differentiation, or a combination of these actions. Clinically, isotretinoin (13-*cis*-retinoic acid) significantly decreases the incidence of second primary tumors in patients with head and neck cancer and also reduces appearance of non-melanoma skin cancer in patients with xeroderma pigmentosum. Retinoic acid has proven to be an effective treatment for promyelocytic leukemia. However, retinoid resistance limits its use as a single agent. Clinical trials are in progress to determine the efficiency of retinoids in treating other types of cancer such as neuroblastoma and breast carcinoma. The development of receptor-selective retinoids and selective inhibitors of retinoid metabolism may lead to further use of retinoids in both chemoprevention and treatment of cancer.

INTRODUCTION

The intention of this review is to discuss recent advances (within the last several years) in our understanding of how retinoic acid, the major physiologically active metabolite of vitamin A, functions as a chemo-preventative agent. Also I will review data on recent clinical trials that have used retinoic acid or synthetic analogs as chemo-prevention and therapeutic agents in selected human cancers. Readers seeking a more comprehensive treatment of this and related fields are referred to the following reviews¹⁻³.

Vitamin A and its metabolites are vital for biological functions such as embryogenesis, growth, differentiation, vision and reproduction⁴⁻⁸. Early studies with vitamin A-deficient animals revealed a connection between this vitamin and susceptibility to carcinogenesis^{9,10}. Subsequently, Lotan and others¹¹⁻¹³ demonstrated that many tumor cells grown in vitro had their proliferation significantly reduced by the addition of retinoic acid to the culture medium. It was also found that retinoic acid could restore “normal” functions (differentiation) to certain tumor cells such as neuroblastoma¹⁴, melanoma¹⁵, and promyelocytic leukemia¹⁶. In the latter case, these laboratory findings led to successful clinical trials such that use of retinoic acid is standard treatment for promyelocytic leukemia. Insight into the mechanism by which retinoic acid is able to reduce carcinogenesis in animals and to inhibit tumor cell growth and/or induce differentiation in vitro took a major leap forward with the discovery of the nuclear receptors in 1989^{17, 18}.

Our understanding of retinoic acid action has made great strides in the area of control of gene expression, but progress in unraveling cellular retinoid metabolism and nuclear transport has been slower. It appears that cellular retinol and retinoic acid binding proteins act as transport proteins to move these retinoids to different cellular locations where metabolism occurs¹⁹. Work by Chytil's group²⁰, suggested that cellular retinoic acid binding protein could be found in the nucleus which implied that it may affect gene function. This concept fell out of favor after the discovery of the nuclear receptors. However, the use of a newer generation of antibodies has revealed that cellular retinoic acid binding protein type II (CRABP II) is found in the nucleus and binds to both RAR and RXR²¹. These data suggest that CRABP II may be the molecule which shuttles

retinoic acid from its site of synthesis in the cytoplasm into the nucleus and delivers this ligand to the nuclear receptors.

It is now well accepted that most if not all of the action of retinoic acid is due to its ability to alter gene transcription. The relevant physiological form of the nuclear receptors consist of an RAR and RXR heterodimer. RAR and RXR each have three subtypes (α , β , and γ)²²⁻²⁴. Gene “knockout” experiments suggest that the RAR subtypes are functionally redundant^{25,26}. However the interpretation of these findings has been question and it was suggested that the experimental conditions create an artificial redundancy²⁷. Knock out of RAR genes in F9 teratocarcinoma cells led to different conclusions, i.e. that the different receptors control specific pathways of RA-induced differentiation^{28,29}. All of the RAR genes can generate isoforms by using alternate promoters, alternate splicing and initiation of translation at an internal CUG codon³⁰⁻³⁴. In contrast, among the RXR, only the RXR γ gene has been found to yield isoforms³⁵. Despite the expression of these isoforms in a tissue-specific fashion, it is not known whether they regulate different sets of genes. RAR specifically binds all-*trans* retinoic acid and 9-*cis* retinoic acid with almost equal affinity, while RXR only has high affinity for binding 9-*cis* retinoic acid³⁶⁻³⁸. RXR can also form heterodimers with other nuclear receptors such as vitamin D3 and peroxisome proliferator-activated receptors^{39,40}. A major difference with these RXR partners is that ligand for either receptor can activate transcription of the target gene⁴¹, while with RAR, ligand cannot bind to RXR due to steric interference⁴². However, after RAR is activated by binding of retinoic acid, the resultant conformational change allows RXR to bind its ligand. When both receptors in the heterodimer bind ligand there is a synergistic increase in target gene transcription⁴³. Activity of the nuclear receptors is also regulated by co-repressors, which bind in the absence of ligand, and co-activators, which only bind in the presence of ligand^{44,45}. One of the key co-activators is CBP/p300, which contains histone acetyl-transferase activity⁴⁶. By acetylating histones and “loosening” chromatin structure, this activator may allow RXR/RAR access to target genes (Fig. 1). CBP/p300 is also required by many other nuclear hormone receptors and transcription factors⁴⁶⁻⁴⁷. Thus RAR activity may be influenced by levels of these other receptors and transcription factors which compete with RAR for the limited amount of CBP/p300 present in the cell’s nucleus.

RETINOIDS AS CHEMOPREVENTATIVE AGENTS: MECHANISTIC AND CLINICAL STUDIES

Retinoic acid is thought to act as an inhibitor of carcinogenesis by interfering with promotion rather than initiation. Promotion can be blocked by a number of different mechanisms, e.g. inhibition of proliferation, stimulation of differentiation, or induction of apoptosis (Fig. 2). Most of the studies describing these effects have been performed on tumor cells rather than pre-malignant cells. The underlying assumption is that the mechanism for retinoid action will be similar in both cell types. However, what appears to be emerging from these studies is that retinoic acid may achieve its physiological effects through different pathways, depending on the origin of the cell type investigated (e.g. skin vs. nerve).

Mechanistic Studies

Growth Arrest: In most sensitive cells, retinoic acid blocks cell cycle progression somewhere in the G1 phase of the cycle. Thus investigators have explored the effect of retinoic acid on cell cycle regulatory proteins. In human neuroblastoma cells which arrest in G1 within two days of retinoid treatment, there was an increase in cyclin-dependent kinase (cdk) inhibitor p27^{Kip1}, but not in p21^{Waf1/cip1}⁴⁸. This increase was coincident with a decrease in cyclin-dependent kinase activity and an increase in G1 cyclin/kinase bound p27^{Kip1}. This RA-induced cdk inhibitor may be crucial for retinoid-induced growth arrest in these neuroblastoma cells. In contrast to this study, a novel mechanism for retinoic acid-induced growth arrest in immortalized human bronchial epithelial cells was reported by Landefeld, et al.⁴⁹. They found that retinoid-treated cells had a marked decline in cyclin D1 protein (required for progression through G1), but not mRNA. It was shown that this was due to enhanced ubiquitin-dependent proteasome degradation of cyclin D1. The mechanism by which retinoic acid enhanced this proteolysis remains unknown. A third potential mechanism for growth arrest was reported by Teixeira and Pratt⁵⁰. They found that in human breast cancer cells (MCF-7) retinoic acid reduced the mRNA level of cyclin D1 and cdk 2, followed by a decrease in their protein levels. The change was specific since there was no alteration in the mRNA

or protein level of cdk 4 and cdc-2 (the mitotic cyclin-dependent protein kinase). Extracts from retinoid-treated cells also contained a cdk 2-inhibitory activity. None of these studies address the mechanism by which retinoic acid changes the level of cyclin, cdk, or cdk inhibitor. From the time course data it appears unlikely that retinoic acid is directly regulating the transcription of these genes. The challenge in the next few years will be to elucidate the pathway which lead to the change in the expression of these proteins.

apoptosis: In addition to inducing growth arrest, retinoic acid and various retinoid analogs can also induce apoptosis in certain cell types. For example, retinoic acid treatment causes apoptosis in human hepatoma cells⁵¹ and a variety of adult T-cell leukemia cell lines⁵². In the hepatoma cells, retinoic acid up-regulated p21^{Waf1/Cip1}, Bax and cdc2 kinase as well as Rb2 and its phosphorylation. An inhibitor of cdc2 blocked the activation of this kinase by retinoids and prevented retinoic acid-induced apoptosis. Retinoic acid also increased the level of p21^{Waf1/Cip1} in the T-cell leukemia lines. Activation of the Fas-Fas ligand system was not involved in retinoid-induced apoptosis. Inhibition of azoxymethane-induced colon carcinogenesis by 9-cis-retinoic acid is accompanied by apoptosis in the adenomas⁵³. A synthetic retinoic N-(4-hydroxyphenyl)retinamide (4-HPR) has chemo-preventative activity in animal models of breast, bladder, lung, ovarian and prostate cancer⁵⁴⁻⁵⁶. It also has a much lower level of deleterious side effects compared to retinoic acid⁵⁵. However, there has been controversy about the mechanism of action of 4-HPR. Oridate et. al.⁵⁷ reported that 4-HPR induced apoptosis by the generation of reactive oxygen species (ROS). Recently, it has become clear that 4-HPR can induce apoptosis by both increasing ROS and also by RAR-dependent mechanisms^{58,59}. In addition, there may be other, as yet unidentified pathways, by which 4-HPR can induce apoptosis⁶⁰. Interestingly, retinoic acid has also been shown to **inhibit** apoptosis in certain cells⁶¹. This finding raises caution about the potential use of retinoic acid as a “universal” chemo-preventive agent for cancer.

differentiation: A third mechanism for the anti-carcinogenic action of retinoic acid is its ability to induce differentiation. This would prevent an initiated cell from being promoted into a tumor cell. In skin carcinogenesis dietary retinoic acid at pharmacological concentrations had little effect on papilloma formation, but inhibited

carcinoma incidence⁶². The mechanism by which retinoic acid can induce differentiation is not yet known. Surprisingly, in some cells a sustained activation of the extracellular signal-regulated (ERK) kinase is required for retinoids to induced differentiation⁶³. Usually this particular mitogen-activated kinase (MAP) is associated with growth factor stimulation of cell proliferation⁶⁴. The difference may be that growth factor stimulation is transient, while retinoic acid treatment induces a sustained activation of the pathway. There are also changes in the retinoic acid response system during carcinogenesis. Skin tumor progression resulted in increased amounts of RXR and decreased amounts of RAR⁶⁵. Also in the conversion of premalignant oral lesions to head and neck cancer, there is a high incidence of loss of RAR β expression⁶⁶. Transfecting normal keratinocytes with oncogenic ras induces a significant reduction in the expression of RAR α and γ receptors⁶⁷. In light of these findings, there is emerging a viewpoint that RAR may function as tumor suppressors.

Clinical Studies

aerodigestive tract: There have been a number of studies showing a statistically significant inverse correlation between beta-carotene (dietary precursor to vitamin A) intake and cancer risk.^{68,69} However in a component of the Physicians Health Study, a 12 year supplementation with beta-carotene or placebo showed no significant difference between the groups with respect to incidence of specific tumors or the overall incidence of malignancies⁷⁰. In head and neck lesions, Hong et al. showed that pharmacological administration of isotretinoin (13-*cis*-retinoic acid) resulted in regression of leukoplakia (pre-malignant lesions)⁷¹. However, the lesions reappeared after the therapy was discontinued. In a more recent study by Hong et al., high dose isoretinoin was found to significantly decrease the incidence of second primary tumors in patients following curative therapy of the initial primary tumor⁷². A clinical trial using N-4-(ethoxycarbophenyl) retinamide found that cancer incidence among patients with severe esophageal dysplasia was reduced by 43.2% compared to patients receiving placebo⁷³.

On the negative side the CARET study (beta-carotene, plus retinol) which included smokers, former smokers, or workers exposed to asbestos was stopped 21 months earlier than planned due to increased risk of lung cancer incidence and mortality in the treated group⁷⁴.

Skin: High dose isotretinoin reduced new skin cancer (non-melanoma) by 63% in patients with xeroderma pigmentosum⁷⁵. In contrast, treatment of patients previously having basal cell carcinoma with low-dose isotretinoin did not produce a significant difference in the occurrence of new basal cell carcinomas⁷⁶. In a moderate risk group (<10 actinic keratoses and no more than two prior skin cancers), treatment with retinol had a significant reduction in the number of squamous, but not basal cell carcinomas⁷⁷. However, the same treatment regimen for a high risk group (history of four or more skin cancers) did not reduce the incidence of either squamous or basal cell carcinoma⁷⁸.

Cervix: Case-control and prospective studies have found an inverse correlation between beta-carotene and presence of intraepithelial lesions in the cervix⁷⁹. However, there was no difference in progression rates of cervical intraepithelial neoplasms (CIN) between patients given beta-carotene vs those given a placebo⁸⁰. Meyskens et al⁸¹ treated patients with grade 2 and 3 CIN with all-*trans*-retinoic acid. With grade 2 CIN, there was complete histological regression of 43% of the treated group vs 27% in the placebo group. No difference in regression rate was found between the two groups with stage 3 CIN (Table 1). These data are quite promising and together with the findings that vitamin A and analogs inhibit human papilloma virus (HPV) - associated proliferation of cervical epithelial cells suggest a place for retinoid therapy in inhibiting the progression of early cervical lesions into cancer.

Breast: There have been observational studies suggesting that breast cancer risk is lower in women having a high dietary intake of vitamin A⁸². Based on promising animal studies with 4-HPR and breast cancer⁸³, a five year study was initiated to determine the efficacy of 4-HPR in reducing the incidence of contralateral breast cancer in patients who had prior surgery for breast cancer. Preliminary results suggest an inhibitory effect of 4-HPR on the occurrence of contralateral breast cancer among premenopausal women⁸⁴. Recent animal studies with a new retinoid analog (targetetin)

suggest that it is more effective than tamoxifen in preventing breast cancer and that it also synergizes with tamoxifen for enhanced chemoprevention activity⁸⁵.

RETINOIDS AS THERAPEUTIC AGENTS: MECHANISTIC AND CLINICAL STUDIES

There are a plethora of studies showing that retinoic acid can inhibit the proliferation and induce differentiation in a variety of human cell lines. Clinical trials to determine the effectiveness of retinoids in treatment of cancer have yielded mixed results. Remissions in patients with promyelocytic leukemia can be induced by retinoic acid with a high frequency. Also neuroblastoma has been shown to have some sensitivity to retinoic acid. However, in many other solid tumors retinoids have minimal or no effect on tumor growth or progression.

Mechanistic Studies

Leukemia: Acute promyelocytic leukemia is invariably associated with chromosomal translocation of the RAR α locus. In 99% of cases, RAR α is fused to the PML gene, which leads to the production of a chimeric protein⁸⁶. PML is an interferon-inducible gene that encodes a protein located in a discrete nuclear structure called PML nuclear bodies⁸⁷⁻⁸⁹. Experiments with mice where the PML gene was inactivated by homologous recombination revealed that PML regulates hemopoietic differentiation, growth and tumorigenesis. These studies also showed that PML is a critical component of the retinoic acid signal transduction pathway and its disruption via chromosomal translocation may lead to development of acute promyelocytic leukemia. Retinoic acid does not appear to have any substantial effect on the growth and differentiation of chronic lymphocytic leukemia (CLL) or acute lymphocytic leukemia (ALL) either in vitro or in a

clinical setting. In contrast, there are some indications that retinoic acid may have a place in treatment of chronic myelogenous leukemia (CML), since it inhibits growth, induces apoptosis and differentiation in CML-like cell lines^{90,91}. Also retinoids have some therapeutic effect especially when combined with other agents in clinical trials (see below).

Neuroblastoma: Sidell first reported that retinoic acid induced growth arrest and differentiation in a human neuroblastoma cell line⁹². Subsequent studies showed that a variety of human and murine neuroblastoma cells were susceptible to retinoic acid-induced differentiation. Following the discovery of the naturally-occurring metabolite 9-*cis*-retinoic acid it was shown that this retinoid had enhanced potency to induce neuroblastoma differentiation as well as apoptosis^{93,94}. One of the characteristics of neuroblastoma is its association with amplified/overexpressed N-myc gene especially in more aggressive disease. Retinoic acid was found to decrease the expression of N-myc and this was synergized by the addition of interferon γ to the cells⁹⁵. Recently, insulin-like growth factor binding proteins (IGFBP) have been implicated in the effect of retinoic acid on neuroblastoma differentiation. Retinoic acid increases the transcriptional rate of the IGFBP-6 gene and reduced the transcription of the IGFBP-4 gene. This change in expression correlates with the postulated roles of these IGFBPs in regulating the growth of neuroblastoma cells⁹⁶.

Breast Cancer: Retinoic acid inhibits mammary carcinogenesis in rodents and inhibits proliferation of human breast cancer cells^{54,97}. Some insight into the mechanism responsible for these actions was found when it was discovered that retinoic acid down-regulates the expression of the progesterone receptor⁹⁸. The same study also found that progestins can down-regulate the expression of RAR α and γ mRNA. A further relationship between steroids, their receptors and retinoic acid was uncovered by the finding that the expression of RAR α is markedly greater in ER⁺ vs ER⁻ human breast cancer cells. Estradiol was found to increase RAR α , but not RAR β or γ expression in an ER⁺ human breast cancer cell line⁹⁹. These findings, i.e. loss of functional RARs, may explain why ER⁺ human breast cancer cell lines are sensitive, while ER⁻ lines are usually

resistant to inhibition of proliferation induced by retinoic acid¹⁰⁰. Some reports suggest that RAR α is the dominant retinoid receptor which mediates the effect of retinoic acid on inhibition of breast cancer cell growth¹⁰¹, while other studies provide strong data that the RAR β receptor mediates the effect of retinoic acid on growth and apoptosis in human breast cancer cells¹⁰².

Investigators have found that overexpression of the c-erbB receptor tyrosine kinase is linked to more aggressive forms of breast cancer¹⁰³. Retinoic acid was found to decrease the expression of c-erbB, specifically c-erbB-2 and 3¹⁰⁴. These results suggest that retinoids might have a role in the treatment of more aggressive forms of breast cancer. Also retinoic acid was shown to inhibit growth in human mammary epithelial cells in which the tumor suppressor p53 was inactivated¹⁰⁵. This is an important finding since many tumors lack functional p53 and drugs that work through a pathway involving this protein are ineffective in these particular tumors.

Clinical Studies

Promyelocytic Leukemia: Retinoic acid has been shown to induce remission with high frequency in patients with acute promyelocytic leukemia (APL)¹⁰⁶⁻¹⁰⁸. Unfortunately, the duration of remission is short (average of 6 months), due to acquisition of resistance to retinoic acid (see below). Recent studies suggest that use of RAR-selective ligands may prolong remission and also reduce potential side effects of this treatment¹⁰⁹. The current standard therapy for APL involves a combination of retinoic acid and chemotherapy. This regimen has resulted in complete remission rates of 90% and has improved long term survival. There is intense interest in the mechanism of retinoid resistance in APL patients. One possible mechanism is accelerated metabolism of retinoic acid. One study reported that a decrease in plasma level of retinoic acid during treatment of APL correlated with clinical recurrence. However, leukemic cells from these patients remained sensitive to the differentiating effect of retinoic acid in vitro¹¹⁰. Substituting 9-*cis*-retinoic acid for all-*trans*-retinoic acid did not result in reversal of resistance despite little change in the plasma level of this retinoid¹¹¹. Recent

studies have examined the mechanism of retinoic acid-resistance in APL cell lines. Mutations were found in the RAR α receptor in several of these cell lines¹¹². It appears that this additional mutation in the PML/RAR α gene blocks the induction of differentiation by retinoic acid¹¹³. Similar mutations were found in cells from APL patients exhibiting retinoic acid resistance¹¹³. Thus resistance to retinoid therapy might have multiple mechanisms making it difficult to attack clinically. Other types of leukemia have not been found to be sensitive to retinoic acid treatment, with the possible exception of CML where some success has been achieved with a combination of retinoic acid and interferon- α ⁹⁰. Also there is some evidence that lymphomas may be susceptible to retinoid therapy¹¹⁴.

Solid Tumors: Although a variety of tumor cells are susceptible *in vitro* to growth inhibition induced by retinoid acid, there has been limited success in the use of retinoids to treat solid tumors (as opposed to chemoprevention). In a small sample of patients with recurrent malignant glioma, there was partial/minor response to high-dose 13-*cis*-retinoic acid in 23% of the group¹¹⁵. Although retinoids combined with interferon α inhibit progression of early cervical lesions (see chemoprevention), this combination has little effect on advanced cervical cancer¹¹⁶. In contrast, this drug combination appears to improve oxygenation of cervical cancer and hence improve the percentage of responses to radiotherapy¹¹⁷. There are several ongoing clinical trials of the effectiveness of retinoids +/- other chemotherapeutic agents on inducing clinical responses in neuroblastoma and breast cancer, but results have not yet been published.

CONCLUSIONS

Results of basic investigations suggest that retinoic acid acts as a chemopreventive agent by inducing differentiation, growth arrest, apoptosis, or a combination of these events. These effects are also responsible for its anti-tumor activity. The pathway(s) by which retinoic acid achieves these effects is still poorly understood. Based on results of clinical trials, it appears that retinoids may have a significant role in the chemoprevention of head and neck, breast, cervical, and some types of skin cancers.

In contrast the only proven effective therapeutic use of retinoids is in the treatment of promyelocytic leukemia. However, the development of receptor-selective retinoids, selective inhibitors of retinoid metabolism and increased understanding of the mechanism of retinoid action may lead to an expanded use of retinoids as therapeutic agents for various forms of cancer.

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Figure Legends

Fig. 1. Schematic diagram of the assembly of a transcriptionally active RXR:RAR complex on target genes.

A. Inactive RXR:RAR complex bound to co-repressor. The RXR ligand binding site is sterically blocked by RAR. Co-repressor has histone deacetylase activity which keeps the chromatin in the “condensed” state. B. Assembly of active complex. Addition of the ligand all-*trans*-retinoic acid, delivered to the nucleus by CRABP II, induces a conformational change in RAR. As a result of the change, the co-repressor is released, the RXR is free to bind its ligand 9-*cis*-retinoic acid, and coactivators such as CBP/p300, pCAF and others (e.g. steroid coactivator-1 (SRC-1)) bind to the RXR and RAR. CBP/p300 and pCAF have histone acetyltransferase activity which induces a “loose” state of chromatin structure required for active gene transcription.

Fig. 2. Mechanism by which retinoic acid inhibits tumor formation.

This diagram shows an initiated cell (carcinogen-induced mutation) being promoted to a small foci of abnormal cells. In the absence of retinoic acid, these abnormal cells can progress to a clinical tumor. When retinoic acid is present, it can either arrest any further growth of the abnormal cells; induce the abnormal cells to differentiate back to their normal counterparts, or induce the abnormal cells to undergo apoptosis. The apoptotic (dead) cells are then ingested by macrophages.

Table 1. The degree of cervical dysplasia affects the ability of retinoic acid to induce regression of CIN lesions.

Number of patients with moderate dysplasia	Regression of lesion with placebo	Regression of lesion with retinoic acid
151	27%	43%
Number of patients with severe dysplasia	Regression of lesion with placebo	Regression of lesion with retinoic acid
150	31%	25%

Data are from a randomized phase III trial conducted by Meyskens and Manetta⁸¹