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Invited review

Biomarker and animal models for assessment of retinoid efficacy in cancer chemoprevention

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Abstract

Vitamin A is essential for normal growth and development. Epidemiology and laboratory studies suggest that decreased vitamin A levels and defective metabolism/action may contribute to the genesis of certain cancers. Based on this information, natural and synthetic derivatives of vitamin A (retinoids) have been used for chemoprevention of cancer. Retinoids have had some success in the chemoprevention of leukoplakia and in the decreased incidence of second primaries in head and neck cancer. There is little information on biomarkers that can be used to assess the efficacy of the chemopreventive activity of retinoids. The ability of retinoids to induce RARb has been consistently shown to correlate with the response of cells and tissues to retinoic acid, but few other biomarkers have been certified as indicators of retinoid activity. In light of the failure of the ATBC and CARET clinical intervention trials for chemoprevention of lung cancer, greater use of animal models for chemoprevention studies is necessary. The potential combination of phytochemicals that inhibit DNA methyltransferase activity with retinoids holds promise for more effective chemoprevention of retinoid-unresponsive premalignant lesions.

Introduction

Vitamin A (retinol) is an essential dietary component. It is required for normal embryonic development, maintenance of growth and differentiation of epithelial cells, male reproductive activity, immune functions and night vision[1–5]. Classic studies by Howe and others[6–8] have established a connection between vitamin A and cell proliferation. Comprehensive studies covering the areas of epidemiology, molecular biology, animal models and clinical science have provided strong evidence that vitamin A, through its metabolites, has tumor suppressor functions. Interest in using vitamin A and its metabolites for the prevention or treatment of cancer began in the 1980s. There have been both successes and failures in this area.

In this review, I will first discuss vitamin A metabolism and molecular function. This will be followed by a summary of our current knowledge of altered vitamin A metabolism and molecular function in cancer cells. Next I will outline the progress in the use of biomarkers to assess the efficacy of retinoids in chemoprevention and cancer, from about 1994 to the present. This will be followed by discussion of various animal models that have or could be used to validate biomarkers. An elaboration of why animal studies are essential before clinical trials will be presented. Finally, I will draw some conclusions from published studies as to our current state of knowledge and suggest areas where high-priority targeted research is needed.

Vitamin A: metabolism and molecular action

We obtain vitamin A (retinol) from our diet because we lack the ability to synthesize this vitamin. Both beta carotene and retinyl esters serve as sources of retinol. Once absorbed by the intestine, retinol is esterified and transported by chylomicrons to the liver. Here it is stored by the stellate cells until it is needed to replenish vitamin A levels. Retinol leaves the liver conjugated to serum retinol binding protein, which, in turn, is bound to the plasma protein transthyretin. The pathway and components of retinol/retinoic acid action

are illustrated in Figure 1. It was recently discovered that cells possess a plasma membrane protein called STRA6 that acts as a cell surface receptor for serum retinol binding protein[9]. This receptor also extracts retinol and delivers it to the inside of the cell for subsequent metabolism. Mutations in STRA6 produce a pleiotropic syndrome that resembles vitamin A deficiency[10]. This finding supports the essential role of STRA6 in retinoid biology. Once retinol is inside the cells, it is metabolized to retinaldehyde and then to retinoic acid. The first step is catalyzed by medium-chain alcohol dehydrogenases, short-chain retinol dehydrogenases and some members of the P450 family. The second step is catalyzed by retinol dehydrogenases and some members of the cytochrome P450 family.

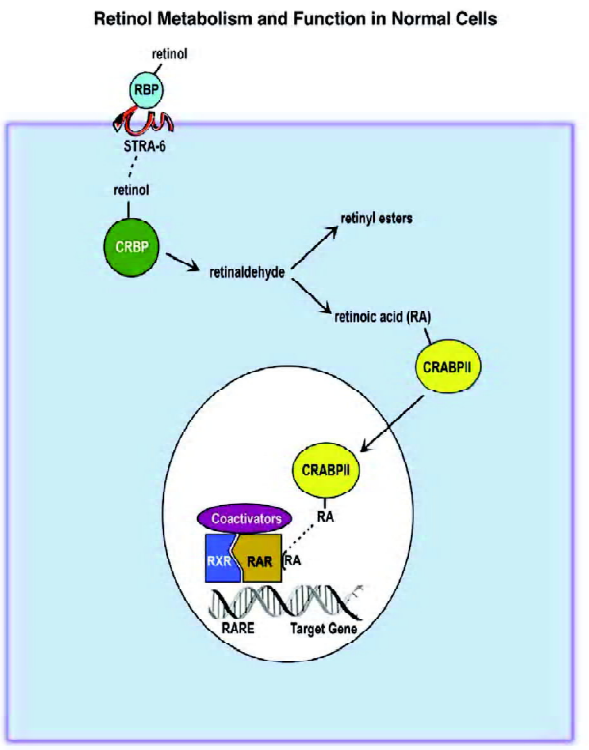

Figure 1. Metabolism and function of vitamin A in normal cells.

Two cytoplasmic retinol binding proteins (CRBP-I and -II) have been described. Likewise, there are two cytoplasmic binding proteins for retinoic acid (CRABP-I and -II). CRBP-II expression is limited to the adult small intestine, while CRBP-I is ubiquitously expressed. Its expression is stimulated by retinoic acid[11,12]. The expression of CRABP-II is somewhat limited, whereas CRABP-I is ubiquitously expressed[13]. Retinoic acid treatment induces CRABP-II expression[14]. The CRBP proteins appear to sequester retinol and shuttle it to different compartments, where it can be metabolized or stored as retinyl esters[15]. In contrast, type II CRABP delivers retinoic acid to the nucleus and can interact with nuclear retinoic acid receptors to alter their activity[16,17].

The most biologically active form of vitamin A is retinoic acid. It appears that most of the biological actions of retinoic acid are mediated by its nuclear receptors. These receptors have been found to contain the same structural modules as a family of steroid hormone receptors. Extensive screening of cDNA libraries revealed that there is a family of retinoic acid nuclear receptors. One class of receptors (RARα, β and γ) binds all-*trans* and 9-*cis* retinoic acid[18], whereas a related class of receptors (RXR α, β, and γ) only binds 9-*cis* retinoic acid with high affinity[19]. In addition, it is known that the RXR form heterodimers with a number of other nuclear receptors, such as RAR[20,21], vitamin D3 receptor[22,23], thyroid hormone receptor[24,25], peroxisomal proliferator-activator receptor[26,27] and a number of orphan receptors[28,29]. Under physiological conditions only the RXR:RAR heterodimer leads to productive DNA binding[30].

These ligand-activated receptors stimulate the expression of target genes through binding to specific retinoic acid response elements (RARE) usually located in the promoter region[31]. The consensus is that RARE is composed of a direct repeat of 5'PuG(G/T)TCA3' separated by five other nucleotides[32]. However, there are a considerable number of RAR target genes whose RARE composition and location vary considerably from the consensus[33]. There are also some genes whose expression is apparently directly inhibited by retinoic acid. The mechanism for this retinoid action is not fully understood[34].

Altered vitamin A metabolism and function in cancer

A number of animal studies have demonstrated that vitamin A deficiency induces an increase in the number of spontaneous and chemically induced tumors[35–37]. The addition of "pharmacological" amounts of vitamin A to the diet reduced the incidence of chemically induced tumors in animals[38–40]. Human epidemiological studies found an inverse relationship between vitamin A/beta-carotene intake or plasma levels and the incidence of several types of cancers, such as lung[41], head and neck[42] and breast[43]. Treatment of acute promyelocytic leukemia patients with retinoic acid induces remission with high frequency. These cumulative studies provide strong evidence that vitamin A and its biologically active metabolites inhibit tumorigenesis. Therefore, in order for cancers to form, the cells must find a mechanism

to subvert the normal biological activity of retinoids. Evidence for these changes is discussed below.

Vitamin A uptake and metabolism are altered in a number of different types of cancer cells (see Table 1). It has been noted that tumor cells have low levels of retinyl esters when compared with their normal counterparts. The enzyme responsible for this esterification, lecithin:retinol acyltransferase (LRAT) is defective in some cancers, whereas in others, CRBP-I expression is lost[44–46]. In addition, retinoic acid is metabolized more rapidly in some tumor cells and the use of chemical inhibitors of the 4-hyroxylase enzyme increases retinoic acid concentration in tumor cells[47]. A number of studies have documented that RAR are altered in cancer cells. Acute promyelocytic leukemia is caused by a translocation of RAR-α resulting in a fusion protein, usually with the promyelocytic leukemia gene[48]. A great deal of experimental evidence implicates RAR-β as a tumor suppressor. Its expression has frequently been found to be reduced or silenced in numerous tumors[49–52]. Cancer cells, which are sensitive to retinoic acid treatment, demonstrate an upregulation of RAR-β when the cells are treated with this retinoid; resistant cancer cells fail to increase RAR-b expression[53]. There is little evidence that the RAR-β gene is lost or mutated in cancer cells. Instead, epigenetic mechanisms play a predominant role in inactivating the function or expression of RAR-β. Two orphan receptors, COUP-TF and nurr77, affect the expression of RAR-β. COUP-TF increases and nurr77 inhibits the expression of RAR-β[54,55]. The relative amounts of these two orphan receptors in cancer cells tracks with their effect on RAR-β expression, that is, low COUP-TF and high nurr77. The major mechanism for RAR-β silencing in cancer is DNA methylation, especially in the promoter region of this gene[56–58]. Treatment of these retinoic acid resistant cells with demethylating agents, such as 5-aza-2'-deoxycytidine, results in re-expression of RAR-β and acquisition of sensitivity to retinoic acid[59,60].

Table 1. Defects in vitamin A metabolism and function in cancer cells.

Defects in retinoid metabolism and function in cancer cells
Decreased uptake of retinol from serum
Decreased conversion of retinol to retinoic acid
Loss of retinyl esters
Decreased expression of CRBP-1
RARα translocation
Silencing of RAR_{β} expression
Methylation of RARE in $RAR_{\beta 2}$ promoter

Retinoids and chemoprevention: biomarkers

Oral premalignancy has been the subject of a number of retinoid prevention trials. The lesions, leukoplakia, are easily monitored and sampled. In 1986, a short-term, high-dose isotretinoin trial was reported. For 3 months, 44 patients received either placebo or 1–2 mg/kg per d isotretinoin. After the cessation of treatment, the patients were monitored for an additional 6 months. Patients receiving isotretinoin had a 67% clinical response rate compared with 10% in patients on the placebo. However, the high amounts of isotretinoin resulted in toxicity and more than 50% of the initial responders relapsed within 2–3 months after the cessation of treatment[61]. Further variations on this protocol included high-dose isotretinoin induction followed by low-dose isotretinoin maintenance[62]. Progression during low-dose maintenance was only 8%. Three other randomized trials with retinoids and oral premalignancy also reported positive results[63–65]. These studies used clinical and histological measures of response. Lotan *et al*[66] used *in situ* hybridization to detect mRNA of RAR in biopsies of premalignant oral lesions. The amount of RAR-β RNA was low in the premalignant lesions and increased in the lesions that responded to isotretinoin treatment. Therefore, in oral premalignancy and other similar states in other tissues, RAR-β is likely to be an important biomarker for retinoid chemoprevention studies.

Lung cancer has been another target of retinoid chemoprevention. A controlled trial of heavy smokers used the metaplasia index from bronchoscopy as an end-point. In a 6-month trial with isotretinoin using 87 subjects, there was a significant reduction in the metaplastic index in both isotretinoin and the placebo subjects[67]. Because of the variability of the metaplastic index, intermediate biomarkers are being sought to incorporate into these clinical trials.

Premalignant skin lesions have also been the target of retinoid chemoprevention studies. These lesions are readily observed and biopsied, making the assessment of chemopreventive activity easy to quantify. Several trials have found that topical[68,69] and systemic[70,71] retinoid treatment resulted in a decrease in the number of actinic keratoses. However, there were relatively small numbers of patients in these studies, and the effects were reversible. Further studies, involving xeroderma pigmentosum patients and renal transplant patients, who are at high risk of developing basal cell and squamous cell skin cancer, respectively, had significantly fewer skin cancers[72,73]. However, the study population was small and the effects were reversible after cessation of retinoid treatment. Several large-scale skin cancer chemoprevention studies using subjects at lower risk for skin cancer

had varying results. One study found a lower incidence of primary squamous cell, but not basal cell, cancer[74], but the other three studies found no significant differences[75–77].

Cervical dysplasia can be followed quite easily and has been the subject of a variety of chemoprevention studies. Advanced trials from the University of Arizona found that tretinoin delivered via a collagen matrix for 1 year resulted in 50% of patients having a decrease in dysplastic lesions[78]. In a follow-up study, tretinoin was found to be more active in reversing moderate dysplasia, but had little effect in patients with severe dysplasia[79].

In these early studies (1980–1994), few attempts were made to identify intermediate biomarkers for retinoid chemoprevention activity. This was probably because of a lack of sensitive techniques/reagents that are needed to routinely quantify markers in relatively small amounts of clinical material. Attempts have been made to define putative biomarkers by examining genes/proteins that interact with or are part of the retinoid pathway in premalignant tissues. For example, Lawrence *et al*[80] found that RXR-α was overexpressed in 66% and 88% of non-comedo DCIS and comedo DCIS lesions, respectively, which are associated with a >8-fold and >12-fold risk, respectively, of developing breast cancer. In contrast, only 8% of lesions that have only a small risk of developing breast cancer had overexpression of RXR-α. Whether the preneoplastic lesions that express high amounts of RXR-α will be more or less susceptible to retinoid treatment is yet to be investigated. Recently, human radial growth phase melanoma cells have been used as a surrogate for screening agents for melanoma prevention. These melanoma cells are the closest to normal melanocytes outside of dysplastic nevi. The markers used for these studies were N-cadherin and P-cadherin. Both 4-HPR and 9-*cis* retinoic acid had some effects on reversing the changes in marker expression after ultraviolet (UV)-B radiation of the cells[81]. It is not clear whether the chosen markers are specific for retinoid chemoprevention activity in melanoma.

A significant amount of progress has been made in defining biomarkers in upper aerodigestive tract cancer. Initiated cells have 9p and 3p loss, whereas mild dysplastic cells have 17p loss, telomerase activation and global DNA methylation. RAR-β loss and EGFR are hallmarks of moderate dysplasia, and severe dysplastic lesions have p53 mutation and increased expression of cyclin D1. Frank carcinoma lesions are associated with increased angiogenesis[82]. In retinoid chemoprevention trials, resistance was associated with abnormal p53 expression, increased degree of genomic instability and lack of RAR-β induction after treatment with 13-*cis*-retinoic acid[83]. However, none of these biomarkers have been validated and it is likely that multiple markers will need to be used to avoid the risk of basing the effectiveness of the chemoprevention agent on the wrong marker. Figure 2 illustrates possible biomarkers for assessing retinoid efficacy in premalignant cells.

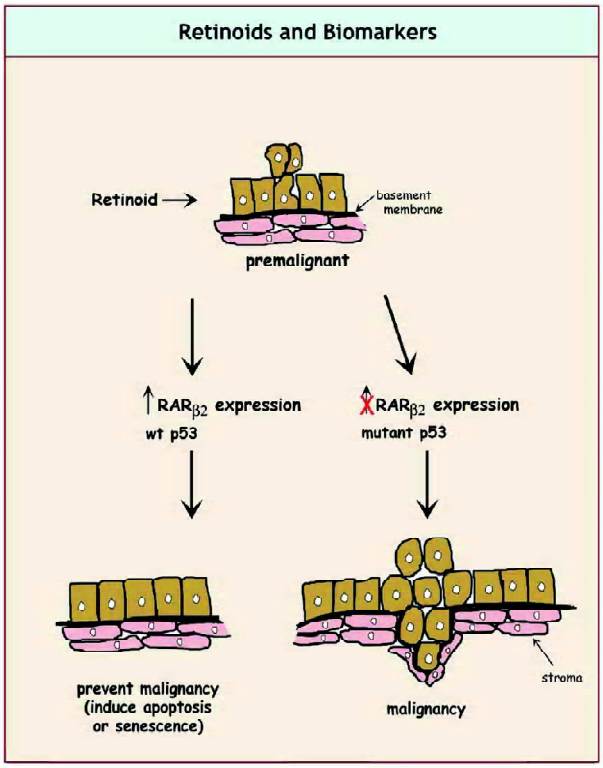

Figure 2. Potential biomarkers for retinoid chemoprevention activity.

Global DNA methylation has been proposed as a biomarker for chemoprevention in colon cancer[84]. Chemoprevention agents that are effective in inhibiting the development of colon cancer decrease colonic DNA methylation, whereas those that are ineffective, including 9-*cis* retinoic acid, do not decrease colonic DNA methylation. Although there have been disappointing clinical primary prevention trials for beta carotene, alpha-tocopherol and retinyl palmitate, a limited trial of 13-*cis* retinoic acid, showed induction of RAR-β expression in lung cancer premalignancy[85]. Whether this will translate to effective chemoprevention of lung cancer in the subset of patients who express this marker is yet to be determined. The most promising marker for retinoid chemoprevention activity is induction of RAR-β expression. This receptor has been found to have reduced or no expression in cancers cells from breast, lung, head and neck, cervix, ovary, melanoma and many other tumor types[86–88]. Tumor

cells that are sensitive to retinoic-acid-induced phenotypic changes (growth inhibition, differentiation or apoptosis) will exhibit a large increase in RAR-β2 expression when retinoic acid is administered[89,90]. In many instances, the reduced expression of RAR-β2 appears to result from increased DNA methylation in the promoter region of this gene[91]. Treatment with DNA demethylating agents, combined with retinoic acid, often leads to restoration of RAR-β2 expression, which is accompanied by growth inhibition[92,93].

Use of animal models to validate biomarkers

Based on the experience of the ATBC and CARET trials, where negative results or increased development of lung cancer were found[94,95], it is imperative that animal trials be used to identify useful chemopreventive agents, intermediate biomarkers and any potential harmful effects. Indeed, subsequent studies with ferrets given the high dose of beta carotene used in the clinical studies and exposed to smoke showed that these animals developed squamous metaplasia[96]. Valid animal models need to develop cancer at the appropriate organ site, with the tumor having the pathological and molecular signatures of the cognate human tumor. Very few carcinogen-induced or transgenic animals pass this stringent test.

The use of retinoids for chemoprevention in transgenic animal models of cancer has been limited. McCormick *et al*[97] found that 4-HPR given after *N*-ethyl-*N*-nitrosurea (ENU) administration to *pim-1* oncogene overexpressing mice, delayed T-cell lymphoma development. However, intermediate biomarkers were not investigated in this study. The C3(1)SV40 large T/*t*-antigen (Tag) transgenic mouse has been developed to model human breast carcinogenesis. These mice develop mammary epithelial dysplasia that progresses to mammary intraepithelial neoplasia, which is similar to ductal carcinoma found *in situ* in humans[98]. At 16 weeks these mice develop invasive carcinoma. Retinoic acid was found to inhibit mammary neoplasia in these mice when treatment was started at 5 weeks of age. A selective RXR analog, LGD1069, also inhibited tumor incidence and multiplicity in this transgenic model[99]. Using the Wistar-Unilever rat model of prostate cancer, McCormick and Rao[100] showed that 9-*cis*-retinoic acid was the most potent inhibitor of prostate carcinogenesis identified at that time (1999). A small number of other studies have shown chemoprevention effects of retinoids in animal models of bladder, pancreas and brain cancers[101–103]. None of these studies investigated intermediate biomarkers that could be used to predict the response of the tumor to retinoid treatment.

Summary, conclusions and future needs

Retinoids have been shown to have chemopreventative activity for a number of tumors. The most studied are upper aerodigestive tract tumors. However, there are no well-defined and accepted intermediate biomarkers to validate the activity of retinoids in preventing tumor formation. Perhaps the marker that shows the most promise is RAR-β2. Its expression and/or inducibility by retinoic acid are decreased in most tumors that have been examined. Methylation of the RAR-β2 promoter has been documented in a number of retinoid-resistant tumors and may account for some of the silencing of this gene. However, because histone deacetylase inhibitors also increase the expression of RAR-β2, other epigenetic mechanisms are likely to be involved in the regulation of its expression. There are now a number of transgenic animal models that, at least partially, recapitulate the development of human cancer. Unfortunately, only a few of these new models have been tested for the ability of retinoids to inhibit tumor formation, and little research has been carried out to identify intermediate biomarkers in situations where

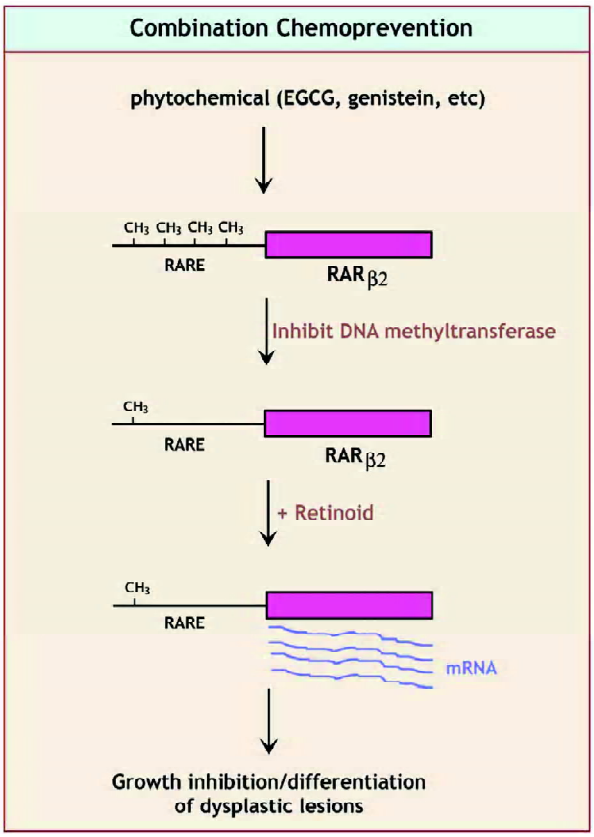

Figure 3. Combination chemoprevention.

retinoids have been shown to be effective. Future needs are to document RAR-β2 and other, perhaps tissue-specific, biomarkers for retinoid efficacy in chemoprevention. This could be accomplished most conveniently with transgenic animal models. In addition, the combination of retinoids (see Figure 3) with phytochemicals that act epigenetically to inhibit DNA methyltransferases or histone deacetylases on cancer chemoprevention is an area that deserves more intense investigation.

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