The Retinoids and Cancer Prevention Mechanisms

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ABSTRACT
Carcinogenesis is a multistep process that converts normal cells into malignant cells. Once transformed, malignant cells acquire the ability to invade and metastasize, leading to clinically evident disease. During this continuum from normal to metastatic cells, carcinogenic steps can be arrested or reversed through pharmacological treatments, known as cancer chemoprevention. Chemoprevention strategies represent therapeutic interventions at early stages of carcinogenesis, before the onset of invasive cancer. Effective chemoprevention should reduce or avoid the clinical consequences of overt malignancies by treating early neoplastic lesions before development of clinically apparent signs or symptoms. Preclinical, clinical, and epidemiological data provide considerable support for cancer chemoprevention as an attractive therapeutic strategy. This clinical approach was validated in the recent tamoxifen randomized trial, demonstrating that a selective estrogen receptor modulator reduces the risk of breast cancer in women at high risk for this malignancy.

INTRODUCTION
Carcinogenesis is a chronic and multistep process, resulting from mutagenic damage to growth-regulating genes and their products, that ultimately leads to development of invasive or metastatic cancers [1]. This transformation from normal through preneoplasia to overt malignancy results from defined steps including: A) initiation, where DNA damage occurs; B) promotion, where additional genetic and epigenetic changes augment prior genomic damage, and C) progression to locally invasive or distant metastatic disease. Carcinogen exposure is hypothesized to form “fields” of altered cells long before invasive malignant disease is detected clinically [2]. Perhaps intermediate markers of carcinogenic changes at affected tissue sites will identify preneoplastic lesions that are likely to progress to a fully transformed phenotype. Alternatively, absence of these markers might indicate epithelial lesions that are unlikely to become malignant transformed.

It is not yet known which individual or cassette of carcinogenic changes are rate-limiting in the maintenance or progression of preneoplastic lesions. Conceivably, these changes are distinct for each epithelial site or carcinogenic agent. Frequent genetic gains or losses are reported to occur at diverse sites, including the head and neck [3], bladder [4], colon [5, 6], lung [7-11], and others. Those carcinogenic pathways required for development or maintenance
of the transformed phenotype at a tissue site may represent attractive therapeutic targets for cancer prevention. Changes in these pathways or of affected dominant oncogenes or recessive tumor-suppressor genes may prove useful to monitor response to clinical cancer prevention agents.

The chronic and multistep nature of carcinogenesis provides a strong rationale for cancer prevention as an attractive therapeutic strategy to arrest or reverse one or more of these carcinogenic changes. This cancer chemoprevention concept was first coined by Sporn [12] and stresses interventions at the earliest stages of carcinogenesis, even before cancers are clinically apparent. If successful, this could avoid many clinical consequences of overt malignancy, as well as the need for treatment of disseminated malignancies that are often less responsive than early neoplastic lesions to therapeutic interventions.

Strong clinical validation for cancer prevention was provided through a randomized trial using the selective estrogen receptor modulator (SERM) tamoxifen in women at high risk for breast cancer development [13]. In those women randomized to receive tamoxifen compared to controls, there was a highly statistically significant reduction in the risk of invasive and noninvasive breast cancers [13]. This reduction was seen for hormone-sensitive breast cancer. Clinical benefits were not seen for hormone-resistant breast cancers. Based on these clinical findings, tamoxifen is now approved by the U.S. Food and Drug Administration for breast cancer risk reduction in high-risk women. This breast cancer prevention trial will be built upon by analysis of other candidate prevention agents, including other SERMs that may have more favorable therapeutic or toxicity profiles than tamoxifen. Effective breast cancer prevention strategies are needed for hormone-resistant breast cancers. One approach taken to address this need is examination of the retinoid N-(4-hydroxyphenyl)retinamide (fenretinide, 4HPR) for prevention of a second breast malignancy in women with early breast cancer [14]. This 4HPR randomized trial reported a potential benefit in premenopausal women for reducing second breast cancers.

The carcinogenic steps of initiation, promotion, progression, and invasion or metastasis can be targeted by antiproliferative, differentiation-inducing, or pro-apoptotic agents, as recently reviewed [15]. Extensive epidemiological, preclinical, and clinical data point to an important role for the retinoids in cancer chemoprevention. This review summarizes how retinoids mediate antineoplastic effects. Emphasis is placed on those retinoid signaling pathways that are responsible for clinical responses. The retinoid role in managing nonneoplastic diseases, regulating immune functions, and causing teratogenic effects is beyond the scope of our review, but is discussed elsewhere [16, 17].

**Clinical Activity of Retinoids**

The retinoids are natural and synthetic derivatives of vitamin A (retinol). They have diverse structures, pharmacological profiles, receptor affinities, biologic activities and specific toxicities [17]. Experimental animal models [18], cellular models [19], epidemiological data [16] and clinical trials [16] provide a strong rationale for the use of retinoids in cancer therapy and prevention. Evidence for the retinoid role in cancer prevention was first provided in 1925 when vitamin A was reported as required for epithelial cell homeostasis [20]. Rats rendered vitamin A-deficient developed squamous metaplasia at several epithelial sites, including the trachea. These tracheal metaplastic lesions were reminiscent of those found in smokers and were reversed following correction of the vitamin A deficiency. An additional link between vitamin A levels and incidence of neoplasia is found through epidemiological evidence showing an inverse relationship between vitamin A levels and incidence of specific malignancies, as reviewed [16].

These and other findings provided a basis for use of retinoids in clinical cancer prevention trials. Added support for a retinoid-based clinical chemopreventive approach stemmed from the successful retinoid treatment of premalignant lesions such as oral leukoplakia [21], cervical dysplasia [22] and xeroderma pigmentosum [23]. Clinical trials reveal that retinoids are active in reducing some second primary cancers. For example, 13-cis-retinoic acid (13-cRA) reduces second aerodigestive tract tumors in patients with resected head and neck cancers [24]. Second primary lung cancers are reduced by retinol palmitate treatment of patients following resection of stage I lung cancer [25]. The acrylic retinoid, polyurenoic acid, inhibits second hepatocellular carcinomas after resection or ablative of primary liver cancer [26].

These findings, when coupled with the single-agent activity of retinoids in treating overt malignancies, including acute promyelocytic leukemia, juvenile chronic myelogenous leukemia and mycosis fungoides, and the successful combination therapy with interferon-α-2A in the treatment of squamous cell carcinoma of the skin or cervix and in renal cancer, as reviewed [16, 19], provide support for a therapeutic role for the retinoids in the treatment of neoplastic disease. Recent evidence that 13-cRA is beneficial in the treatment of high-risk neuroblastoma after bone marrow transplantation indicates how the retinoids may have an adjuvant therapeutic role in the management of minimal residual disease in responding malignancies [27].

Vitamin A-associated toxicities limit chronic administration of retinoids to individuals at high risk for cancer development [21, 24]. Epidemiological evidence for an inverse relationship between serum vitamin A or β-carotene levels and specific cancer incidences led to cancer prevention trials.
using β-carotene because it is clinically well-tolerated when chronically administered. However, randomized trials using β-carotene for primary lung cancer chemoprevention did not yield a reduction in lung cancers in high-risk individuals [28-30]. Indeed, in the treated versus control group, there appeared to be an even higher lung cancer incidence, perhaps due to the continued smoking history of affected individuals [28]. Similar effects may occur in current smokers who are treated with 13-cRA to prevent second primary tumors [31].

These clinical findings emphasize a need for additional basic scientific studies to identify prevention mechanisms, especially in those individuals who no longer smoke. These would help optimize the conduct of clinical cancer chemoprevention trials that are often of long duration and costly. This also underscores the potential value of using in vitro carcinogenesis models to determine intermediate markers of transformation and select appropriate agents for testing in clinical cancer prevention trials. To understand how retinoids are active in cancer chemoprevention, it is important to review those mechanisms that signal their biological effects.

**Mechanisms of Retinoid Action**

Basic scientific studies have highlighted key regulators of the retinoid signaling pathway. Retinoids signal cellular effects through nuclear retinoid receptors and their coregulators, as recently reviewed [19, 32]. This leads to ligand-dependent transcriptional activation of target genes that ultimately signal retinoid growth and differentiation effects. Two classes of nuclear retinoid receptors are identified. These are the retinoic acid receptors (RARs), and retinoid X receptors (RXRs), respectively. They share sequence homology with other members of the steroid receptor superfamily including the vitamin D receptor, glucocorticoid receptor, estrogen receptor, and others [33]. The RARs have three subtypes with several isoforms: RARα, RARβ, and RARγ [32, 34-37]. Three RXRs exist: RXRα, RXRβ, and RXRγ [38-41]. There are also orphan nuclear receptors for which physiologic ligands are being identified.

Retinoids bind their nuclear receptors through ligand-binding domains. The retinoid nuclear receptors contain DNA-binding domains that recognize specific sequences present in genomic DNA. As a result of these ligand-receptor and receptor-DNA interactions, direct retinoid target genes that contain retinoid response elements in their promoter regions become transcriptionally activated or repressed. These ultimately lead to changes in gene expression [31, 32], that mediate biological effects. The retinoid nuclear receptors can form homodimers or heterodimers [32]. RARs heterodimerize with RXRs [32, 42]. RXRs heterodimerize with multiple members of the steroid receptor superfamily. Heterodimerization represents an important level of regulation for nuclear receptor-dependent signaling pathways [32, 42, 43]. For example, RXR homodimers are reported to preferentially form over RXR heterodimers following 9-cis-retinoic acid treatment [44]. Retinoid biological signals depend on the type of cells studied. Targeting of individual retinoid receptors reveal receptor-specific developmental and differentiation defects [32, 42, 45, 46]. Two classes of proteins also exist that interact with nuclear receptors through protein-protein interactions. These are known as coregulators and include inhibitory corepressors and stimulatory coactivators. These coregulators also contribute to retinoid signaling [47, 48]. These protein-protein interactions play an important role in how retinoid receptors affect the basal transcriptional machinery [49].

Cytosolic retinoid-binding proteins exist, including the cytosolic retinoic acid-binding proteins and the cytosolic retinol-binding proteins. These appear to contribute to the retinoid metabolism and signaling pathways by regulating intracellular binding of retinoids. These cytosolic receptors may serve as intracellular storage sites for the retinoids that facilitate retinoid transport from the cytoplasm into the nucleus [17, 19]. Induction of cytosolic retinoid receptors may account for clinical retinoid resistance, as reported in acute promyelocytic leukemia [50], although pharmacologic mechanisms may also contribute to this resistance [51].

An improved understanding of mechanisms of retinoid actions has resulted from studies using pharmacological agonists and antagonists for specific nuclear retinoid receptors. It is known that 9-cis-retinoic acid is a bifunctional retinoid activating both RAR and RXR pathways, while all-trans retinoic acid (RA) activates only the RAR pathway [52, 53]. Retinoids exist that are agonists or antagonists for specific retinoid receptors [54, 55]. These have been studied during induced tumor cell differentiation [56, 57]. Other retinoids antagonize the transcription factor AP-1, a key regulator of cellular growth and differentiation [58]. Another retinoid, fenretinide, N-(4-hydroxyphenyl)retinamide (4HPR), preferentially signals apoptosis through receptor-independent mechanisms [57, 59, 60]. However, 4HPR is reported to transcriptionally activate RARγ [61] and to upregulate RARβ expression in several tissue types [62]. In certain cell contexts, 4HPR induces reactive oxygen species [60, 63]. 4HPR triggers apoptosis even in RA-resistant tumor cells [57]. This is consistent with the view that 4HPR mediates its biological effects through mechanisms that are independent of retinoid nuclear receptors.

In the myeloid leukemia cell line HL-60, retinoids cause activation of mitogen-activated protein kinases (MAPKs), such as ERK2, that are necessary for RA-induced growth arrest, cellular differentiation and hypophosphorylation of the Rb protein [64]. Differentiation of F9 embryonal carcinoma
cells into primitive endoderm by retinoids is accompanied by an increase in endogenous \( ras \) and ERK activity [65]. Induction of \( RAR\beta \) in human bronchial epithelial cells is regulated by MAPK-dependent signaling pathways. Phosphorylation by MAPK either inhibits the activity of retinoid receptors directly or through a distinct inhibitory factor. The net result is inhibition of bronchial epithelial cell differentiation [66].

**RETINOID RECEPTOR EXPRESSION AND RETINOID RESPONSE**

A tight relationship exists between expression of specific retinoid receptors and retinoid clinical responses. For instance, in acute promyelocytic leukemia, expression of the \( t(15;17) \) fusion transcript, PML/RAR\( \alpha \), predicts clinical retinoid response [67]. In oral leukoplakia, an association exists between retinoid-induced \( RAR\beta \) mRNA expression and clinical response to 13-cRA [68]. After successful treatment of oral leukoplakia with 13-cRA, \( RAR\beta \) mRNA expression is induced preferentially in clinically responding lesions.

Associations exist between basal or induced retinoid receptor expression and retinoid responses in model systems. Studies of retinoid-resistant HL-60 myeloid leukemia cells that express an altered \( RAR\alpha \) revealed that \( RAR\alpha \) transfection overcomes retinoid resistance in this leukemic cell line [69]. Other studies indicate that \( RAR\gamma \) regulates retinoid growth and differentiation responses in cultured human embryonal carcinoma cells [57, 70]. \( RAR\beta \) is a major mediator of retinoid growth suppression in squamous cell carcinoma cells [71]. \( RAR\beta \) and RXR are involved in growth inhibition of immortalized and transformed human bronchial epithelial cells [72, 73]. These and other studies provide a basis for use of retinoid nuclear receptor-selective agonists or antagonists in future chemopreventive or therapeutic trials. Perhaps combination regimens using receptor selective agonists in conjunction with other chemopreventive agents will exhibit cooperative clinical effects while reducing retinoid-associated toxicities.

**IN VITRO MODELS FOR RETINOID CHEMOPREVENTION**

The optimal retinoids for use in clinical cancer chemoprevention trials need to be determined. If clinical outcome is the only endpoint used for chemoprevention activity, then progress in this field will not be rapid. One approach to assess activities of candidate chemoprevention agents is to examine their effects in relevant in vitro models before their entrance into prevention trials. The mechanistic insights that are derived should aid in the conduct of cancer chemoprevention trials.

Epithelial cell transformation can be prevented in vitro by retinoid treatment [74]. The BEAS-2B human bronchial epithelial cell line has been adapted to investigate carcinogenic transformation, as depicted in Figure 1. This immortalized human bronchial epithelial line was derived using an adenovirus 12-SV40 hybrid virus [75]. These cells were transformed after exposure to tobacco-derived carcinogens [74], such as cigarette smoke condensate or N-nitrosamine-4-(methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK). Notably, RA treatment inhibited the carcinogenic transformation of these epithelial cells [74]. This model has proven useful to identify transformation pathways activated by carcinogens that can be antagonized by treatment with retinoids or other prevention agents [73, 76]. It also expedites structure-function analyses to select the optimal retinoid for use in prevention of transformation of human bronchial epithelial cells [73].

**A RETINOID CHEMOPREVENTION MECHANISM**

The in vitro chemopreventive activity of retinoids in human bronchial epithelial cells has been linked to the triggering of G\(_1\), cell cycle arrest, a concomitant growth suppression, and a decline in expression of G\(_1\) cyclin proteins [73, 74, 76, 77]. This retinoid-triggered G\(_1\) arrest is due to a posttranslational mechanism, as shown in Figure 2A. This retinoid repression of G\(_1\) cyclin expression is blocked by inhibitors of the proteasome-dependent degradation pathway [73, 76, 77] as depicted in Figure 2A. This finding of retinoid-mediated cyclin D1 proteolysis indicates that proteasome-dependent degradation mechanisms are active in the prevention of cellular transformation by the retinoids. This delay at G\(_1\) signaled by retinoid treatment permits repair of mutagenic damage to genomic DNA by carcinogens, as summarized in Figure 2B.

When normal, immortalized, or carcinogen-transformed human bronchial epithelial cells are treated with receptor-selective retinoids, \( RAR\beta \) and RXR-dependent pathways preferentially signal growth suppression [73]. In marked contrast,
α-carotene or β-carotene are unable to repress cyclin D1 protein expression or activate this proteasome-dependent degradation pathway [73]. These effects are consistent with prior reports of inactivity of carotenoids in lung cancer prevention [28-30]. RARβ and RXR agonists, unlike other retinoid receptor-selective agonists examined [73], induced the proteasome-dependent proteolysis pathway previously shown to be activated by RA treatment. Future studies should explore structure-function analyses using other known or candidate prevention agents to learn whether the retinoids are the optimal agents that signal this degradation of G1 cyclins. These findings illustrate the utility of an in vitro model to assess the activities of candidate prevention agents before their entrance into clinical prevention trials. Whether this in vitro prevention mechanism is activated in vivo in the bronchial epithelium during retinoid treatment is not yet known.

Findings reveal that proteolysis of G1 cyclins with concomitant growth arrest is a retinoid chemoprevention signal found in normal, immortalized, and transformed human bronchial epithelial cells [73, 74, 76]. It is recently reported that retinoids promote ubiquitination of cyclin D1 during induced tumor cell differentiation [77]. This suggests that ubiquitin-dependent proteolysis of G1 cyclins is a common retinoid mechanism responsible for G1 arrest. An implication of these in vitro results is that either cyclin D1 or cyclin E would be aberrantly expressed in bronchial preneoplastic lesions. This hypothesis is supported by immunohistochemical studies that indicate these G1 cyclins are frequently aberrantly expressed in bronchial preneoplasia [7]. Perhaps these cyclins will be useful intermediate markers for future lung cancer prevention trials.

CONCLUSIONS

There is a convergence of basic scientific and clinical findings in the retinoid field. While clinical retinoid activity is reported in treating certain premalignant lesions and reducing second primary tumors of the aerodigestive tract or liver, the optimal retinoid useful in primary cancer prevention in high-risk individuals is not yet known. Associations exist between clinical retinoid responses and expression of specific nuclear retinoid receptors. This indicates how specific retinoid receptor-dependent transcriptional pathways appear to transmit retinoid biologic effects. These findings provide a basis for the use of retinoid receptor-selective agonists in cancer therapy or prevention. Perhaps these nuclear receptor-selective retinoids will have more favorable toxicity profiles than nonselective agonists. One way to guide development of future cancer prevention strategies using the retinoids is to explore chemopreventive activities in relevant in vitro models. These models should help identify important pathways responsible for chemopreventive effects and highlight new therapeutic targets for chemoprevention. An example of this is the retinoid regulation of G1 cyclins found in human bronchial epithelial cells [73, 76]. This has already led to the discovery of frequent aberrant expression of G1 cyclins in bronchial preneoplasia [7].

While attention in this review has focused on retinoid chemopreventive activities, there are pharmacological agents affecting other pathways that are also important in cancer prevention [15]. For example, SERMs [78] and inhibitors of cyclooxygenase-2 (Cox-2) [79], are under study in clinical prevention trials. Other candidate cancer chemoprevention agents, such as the triterpenoids [80] are undergoing extensive preclinical investigations. Combination therapy involving the retinoids and other chemoprevention agents is an attractive
pharmacological strategy [15]. Indeed, retinoids have already been shown to cooperate with interferon-α-2a in treatment of squamous cell cancers of the skin and cervix, as well as in kidney cancer [81-83]. Perhaps these or other combination regimens will be beneficial in clinical cancer prevention. Alternate strategies for drug delivery may offer additional advantages. For instance, aerosolized delivery of retinoids or other prevention agents may enhance the treatment of aerodigestive tract malignancies. Candidate cancer prevention agents with potent activities but dose-limiting systemic toxicities may become available for clinical use in this setting when administered via aerosolized delivery [84]. In summary, an improved understanding of the mechanisms of action of the retinoids and other prevention agents should aid in the design and conduct of clinical cancer prevention trials.

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