Minireview

Retinoid Chemoprevention of Aerodigestive Cancer: From Basic Research to the Clinic

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Abstract

Epithelial cancers in the lung and head and neck are a devastating group of diseases which account for approximately 35% of cancer deaths in the United States. Chemoprevention is a new strategy to block or reverse the carcinogenic process. Biological concepts including field carcinogenesis and multistep carcinogenesis strongly support the rationale for using chemopreventive approaches. Our group has focused on applications of translational retinoid studies to increase our knowledge of the molecular events in biology and chemoprevention. In this review, we will focus on four issues, biology, retinoids, retinoid clinical trials, and translational research, in the chemoprevention of aerodigestive cancers.

Introduction

In contrast to the cytotoxic treatment for invasive cancers which are intended to kill cancer cells, chemoprevention is designed to block the initiation and promotion phases of the carcinogenic process, or to reverse the premalignant process before it progresses to cancer. Chemoprevention goals can be achieved at three different levels. At the clinical level, cancer incidence should be decreased. At the tissue level, the reversal of the premalignant phase must be detected. At the cellular level, abnormal clones must be eradicated. Therefore, understanding the biology of carcinogenesis is crucial to the development of successful chemoprevention. Aerodigestive cancers are an important target for chemoprevention because improvements for these common solid tumors in terms of survival have been only marginal in the last two decades, despite improvements in multimodality therapeutic techniques (1).

Three important basic concepts support the role of chemoprevention in controlling epithelial cancers. These are: multistep carcinogenesis, diffuse field carcinogenesis, and retinoid intervention. Aerodigestive cancers are typically a product of the multistep process and field defects due to continuous carcinogen exposure (2). Genetic damage appears to accumulate during the carcinogenic process, and specific genomic alterations have been identified. Mutated or altered p53 is detected in approximately one-half of early epithelial cancers in humans and is also associated with genomic instability. The field carcinogenesis theory proposes that multiple preneoplastic lesions of independent origin occur in the epithelial field as a result of repeated exposure to carcinogens, leading to the associated high incidence of second cancers within the aerodigestive tract that arise in patients with primary head and neck cancer. Discordant p53 mutations in corresponding second tumors arising in patients with primary head and neck cancer suggest that these cancers are independent events. The retinoid anticarcinogenic approach has been strongly supported by results from laboratory studies in animal models as well as epidemiological findings (3).

Controlled clinical trials are the foundation of chemoprevention programs. Over the last 10 years, our group has conducted a series of randomized chemoprevention trials of the synthetic retinoid 13cRA in aerodigestive cancers. Very encouraging results from several retinoid trials (13cRA, fenretinide in oral leukoplakia, and 13cRA and retinyl palmitate trials for prevention of second primary tumors) have recently increased enthusiasm for the potential of this approach in reducing the incidence of aerodigestive epithelial cancers (3).

Despite the scientific rationale for chemoprevention approaches and some proven efficacy, there are still many problems to be solved through translational research efforts. Further advances in molecular epidemiology and molecular genetics in epithelial carcinogenesis will increase our understanding of biomarkers as intermediate end points for chemoprevention trials. Recent new findings in respect to the suppression of RAR-β in oral carcinogenesis and the up-regulation of RAR-β after 13cRA treatment, as well as the correlation of p53 expression and retinoid activity, will be discussed in this review.

Basic Science Aspects of Aerodigestive Carcinogenesis

The development of new chemopreventive strategies for aerodigestive tract tumors requires both a better understanding of the tumorigenesis process, and the development of biomarkers which can be used in both risk assessment and as intermediate markers of response to intervention (3, 4). Two general themes have developed that form the basis for cellular studies on human aerodigestive tract carcinogenesis. First, it has been...
proposed that aerodigestive tract cancer represents a field cancerization process whereby a whole tissue field is at risk for tumor development due to extrinsic and/or intrinsic etiological factors. Second, it has been proposed that aerodigestive tract tumors arise through a multistep process whereby an accumulation of specific genetic changes are translated into phenotypic changes which characterize malignant evolution. These concepts have important implications for chemoprevention studies.

Field Cancerization. The working hypothesis that aerodigestive tract cancer represents a field cancerization process is supported by several types of evidence. First, the tumors occur in a tissue of high carcinogenic exposure. For example, the use of tobacco products greatly increases tumor risk in the exposed field. Tumors can occur throughout the aerodigestive tract, and the risk is associated with the extent and duration of tobacco exposure. Moreover, concurrent exposure to additional etiological agents (e.g., alcohol) can significantly increase the risk within the exposed fields (e.g., oral cavity). Second, multiple tumors occur at an increased frequency throughout the exposed field.

Support for the field cancerization theory can also be derived from pathological examinations of the tissue at risk. For example, in the late 1950s, Auerbach et al. (5) carried out an extensive histological examination of the lungs of more than 100 male patients dying with or without lung cancer. In tissues derived from most of the light smokers and in all of the heavy smokers and individuals with lung cancer, 90–100% of the sections examined showed evidence of some epithelial change, ranging from loss of cilia and basal cell hyperplasia to carcinoma in situ. These changes were present throughout the lung field, and the degree of change correlated strongly with the degree of tobacco exposure.

Most carcinogens are known to cause genetic damage. Thus, a number of groups have initiated studies to explore the nature and extent of the tumorigenic field using cellular and molecular probes. For example, micronuclei (resulting from chromosome nondisjunction during cell division due to chromosome damage) can be detected at relatively high frequencies in exfoliated cells of the aerodigestive tract (obtained by bronchial or oral brushings) of tobacco-exposed individuals (4). The fact that these exfoliated cells have a limited life time and rapid turnover rate would suggest that genetic instability is an ongoing process in the exposed tissue. If genetic instability were an ongoing process associated with continued carcinogen exposure, and tumors can develop anywhere in the exposed field, then one might expect to see an accumulation of genetic damage throughout the exposed tissue field. Our own group addressed this possibility by using the technique of premature chromosome condensation to visualize the interphase chromosomes of cytologically “normal” lung cells obtained at the time of pneumonectomy for lung tumors (6, 7). We observed that lung cells away from the site of the tumor harbored genetic changes that took several forms. First, there were cells exhibiting varying numbers of chromosomes per cell, suggesting a pattern of generalized genomic instability in the exposed normal lung tissue. Second, there were specific karyotypic changes, some of which were held in common with the tumor and some of which were distinct from the pattern found in the tumor. These results again suggest that genetic changes are accumulating throughout the exposed field. The finding of some common cytogenetic changes between the tumor and the normal tissue either suggests that specific genetic changes might be important for the survival of damaged cells or selective outgrowth, or that some of the cells found in the normal tissue have a common clonal origin within the tumor. The finding of distinct cytogenetic changes in the normal tissue suggests that damage continues to accumulate in the normal tissue, thus increasing the risk that important genetic loci will be altered and multiple primaries may develop.

More recently, the notion of field cancerization has been addressed by characterizing molecular events that have occurred in the field of tumors. A number of specific genetic changes involving oncogenes and tumor suppressor genes in aerodigestive tract tumors have been identified, and probes for these changes have been utilized to determine whether some of these same changes have occurred elsewhere in the field. For example, Sundaresan et al. (8) reported that regions of bronchial dysplasia adjacent to invasive squamous cell carcinoma of the lung showed the same molecular change as was found in the tumor. Similarly, Gazdar (9) has microdissected premalignant epithelial regions from lung tissues of cancer patients, and, utilizing PCR methodologies and microsatellite primers and probes, demonstrated a variety of clonal changes, including ras mutations and allelic losses. Sozzi et al. (10) and Lee et al. (11) looked further into the field by examining short-term cultures of normal lung cells from patients with lung tumors and found evidence for chromosomal rearrangements, chromosome aneuploidy (e.g., trisomy 7), and chromosome deletions (e.g., 3p and 17p deletions). Interestingly, the frequencies of these changes appeared to be significantly higher in patients with multiple tumors in the aerodigestive tract. The finding that specific molecular events (e.g., site of p53 mutation, extent of deletion associated with loss of heterozygosity) are usually distinct between the multiple primary tumors (12) and even between different premalignant foci lends further support to the notion that genetic changes are occurring independently throughout the aerodigestive tract, leading to a multifocal tumorigenesis process.

Multistep Tumorigenesis. The second general working hypothesis regarding tumorigenesis in the aerodigestive tract is that it is a multistep process whereby genetic events accumulate which result in phenotypic changes seen as dysregulatory processes that affect cell growth, differentiation, cell loss, and cell function. From a clinical view, tumors of the aerodigestive tract are often preceded by abnormal lesions in the field at risk (e.g., oral leukoplakia/erythroplakia, bronchial metaplasia/dysplasia, Barrett’s esophagus). While tumors do not necessarily originate from these abnormal lesions, the presence of lesions in the field implicates a higher tumor risk somewhere in the field. For example, individuals with oral leukoplakia/erythroplakia demonstrating dysplasia have upward of 30–40% long-term risk of developing head and neck cancer (3).

Our group and others have examined in more detail some of the cellular and molecular changes which accompany the multistep tumorigenesis pathway in an attempt to identify biomarkers that might be useful in assessing tumor risk and response to chemopreventive intervention. To accomplish this, we have identified a number of head and neck tumor samples which contain premalignant tissue adjacent to the tumor such that there
appears to be a contiguous progression of events from histologically normal tissue through hyperplasia and dysplasia to tumor. Tissue sections from these informative specimens were then analyzed for genetic and phenotypic changes associated with tumorigenesis. To examine generalized accumulation of genetic changes, we used the technique of chromosome in situ hybridization utilizing probes that recognize repetitive DNA sequences in the centromeric regions of chromosomes 7, 9, and 17 (13). Regardless of the particular probe used, we observed a sequential increase in the degree of chromosome polysomy or random chromosome gains and losses as the tissue passed from normal histology through progressive carcinogenic phases. Of importance, over one-third of the samples examined showed evidence of polysomy even in the histologically normal epithelium adjacent to the tumor, and polysomy was evident in two-thirds and more than 90% of the cases of hyperplasia and dysplasia, respectively. Similar observations were made for all three chromosome probes, suggesting that increasing generalized genetic instability and accumulation of genetically altered cells can be used as markers of the tumorigenesis process even in the absence of histological abnormality or knowledge of the specific genetic changes which are accumulating in a clonal fashion.

Accumulation of genetic changes during the multistep tumorigenesis process is likely to cause sequential phenotypic changes which are associated with the tumor phenotype, including alterations in cell growth, differentiation, and cell loss capabilities. To address this assumption, tissue sections of tumor specimens containing adjacent premalignant lesions were examined for several phenotypic markers which might characterize the multistep process. The use of an antibody to PCNA on tissue sections allowed visualization of cells that have entered into the proliferative cycle. In biopsies from oral mucosa of noncancer, nonsmoking normal controls, the fraction of PCNA-positive cells is relatively low and confined to the basal layer (14). In contrast, histologically normal epithelium adjacent to head and neck cancers show increased frequencies of PCNA-positive cells, extending into the suprabasal layer, suggesting an early defect in shutting proliferation down beyond the basal layer (possibly reflecting loss of tumor suppressor gene function). The transition toward tumor is then characterized by progressively increased dysregulation of proliferation associated with increased frequencies of PCNA-positive cells in the basal layer as well as extending into the superficial layers.

The p53 gene is frequently found to be mutated or dysregulated in aerodigestive tract tumors and is thought to function as a tumor suppressor gene both in cell cycle checkpoint control and in one of the apoptotic pathways. Since wild-type p53 protein has been suggested to have a short half-life in most tissues, immunocytochemical detection of p53 protein in tissues has been associated with either a mutated p53 gene or altered p53 protein function. Immunocytochemical detection of p53 has been observed in approximately one-half of lung and head and neck cancer specimens examined. Of interest, abnormal p53 expression is detected in histologically normal epithelium adjacent to head and neck tumors in one-fourth of the cases (usually in those cases where abnormal p53 expression is found in the tumor), and this frequency increases to 45% of the cases by dysplasia (15). Associated with this progressive increase in p53 immunostaining is a spatial reorganization of p53 expression from the basal layer into the parabasal and superficial layers in hyperplasia and dysplasia. The pathophysiological consequences of loss of p53 function are not well understood. However, it has been proposed that in chronically exposed tissues such as sun-exposed skin, loss of p53 function can lead to the outgrowth of clones of cells that are resistant to damage-induced apoptosis. If damaged cells in a tissue are not eliminated, this will mean that the rate of accumulation of genetically altered cells in a tissue will increase with continued exposure. This may explain our recent finding that the levels of chromosome polysomy detected in premalignant lesions adjacent to tumors are higher in p53-positive than p53-negative lesions.

Another mechanism for loss of growth control during tumorigenesis is through the inappropriate expression of factors which act in a positive fashion for cell growth. For example, high expression of EGFR has been demonstrated in more than 85% of aerodigestive tract tumors. To determine when during tumor development EGFR expression is increased, tissue specimens containing adjacent premalignant lesions were probed with an antibody to EGFR (16). The relative staining intensity of EGFR in histologically normal epithelium adjacent to head and neck tumors was found to be 2-fold higher than in normal control epithelium, and the degree of EGFR expression increased with carcinogenic progression. Interestingly, the degree of EGFR expression increased sharply from dysplasia to squamous cell carcinoma, possibly suggesting that dysregulation of EGFR expression involves multiple steps. A similar progression of events has been described in other regions of the aerodigestive tract (e.g., esophagus).

**Clinical Implications.** The results stated above provide strong cellular and molecular evidence that aerodigestive tract tumorigenesis involves a field cancerization process that proceeds in a multifocal fashion through a multiple set of events. Moreover, identification of the events important in the tumorigenesis process allows the development of biomarkers which can be used to assess the process in tissue specimens from individuals at risk.

These findings have tremendous implications for chemoprevention studies. For example, in trying to identify the level of cancer risk in a carcinogen-exposed individual, the tissue field at risk is so large that it is difficult to know which tissue site to sample. Thus, one needs to develop markers that can detect evidence for elements of the tumorigenesis process in randomly biopsied material. Since one of the hallmarks of tumor development is the accumulation of genetic abnormalities, one can examine the individual for genetic clues for likelihood of a more rapid accumulation of genetic changes per unit of carcinogen exposure. For example, individuals may differ in their intrinsic ability to handle carcinogenic exposure. Along these lines, a number of investigators have found that peripheral blood cells from individuals with certain kinds of cancer appear to be inherently more sensitive to chromosome-damaging agents such as bleomycin and ionizing radiation. In particular, individuals with head and neck cancers show increased mutagen sensitivity compared to noncancer controls, and among these, individuals with a propensity for developing second primaries show the highest degrees of mutagen sensitivity (17, 18). Interestingly, these individuals appear to show relative hot spots for chromosome breakage sites, and these sometimes appear to overlap...
with sites thought to harbor important genes for tumor development (19). This observation is even more intriguing in light of recent molecular studies suggesting that the same alleles are deleted in different regions of the tumor-associated field, even though these lesions are thought to have developed through genetically independent pathways (i.e., the extent of deletion is different in different lesions). This raises the possibility that inherent sensitivity also involves the predisposition of relevant genetic loci for instability.

If aerodigestive tract cancer is indeed a field cancerization process, and if the degree of genetic change accumulation is associated with the tumorigenesis process, then an assessment of the degree of ongoing genetic or phenotypic change might be useful as a biomarker for risk assessment. Along these lines, our group has retrospectively analyzed biopsies obtained from individuals with oral leukoplasia/erythroplakia for evidence of chromosome polysomy. We have found that individuals with biopsies exhibiting the highest levels of genetic change were at the highest risk for developing head and neck cancer (20). Importantly, nearly one-half of the tumors that developed in these individuals occurred away from the area of biopsy, suggesting that because aerodigestive tract tumors involve a field cancerization process, characterization of a genetic instability process in one part of the tissue field may have risk implications for events happening throughout the field. This may also explain our recent finding that individuals whose primary head and neck cancers exhibit positive p53 expression are at a significantly increased risk for developing early second primaries in addition to having an increased rate of tumor recurrence.

The identification of genotypic and phenotypic events associated with tumorigenesis also has implications for chemoprevention trials. For example, once specific pathways for dysfunction associated with tumorigenesis are identified, then strategies can be developed to target the dysfunction. For example, if loss of the capability to remove damaged cells from an exposed tissue is an early event during tumorigenesis, strategies can be sought to either return this function to the tissue or to selectively target cells which have lost function. Biomarkers of the multistep process can also be used as intermediate markers of response. For example, if a particular chemopreventive strategy was targeted to reduce the accumulation of genetic changes (e.g., antioxidants) or slow proliferation, examination of biopsies taken pre- and postchemopreventive intervention could serve to determine whether the chemopreventive agent was having an effect in the tissue and, if so, along the presupposed pathway predicted by preclinical studies. Similarly, if the chemopreventive strategy was to selectively eliminate a dysfunctional premalignant clone, biomarkers of the tumorigenesis pathway could be used to determine whether a clinically regressed lesion represented selective elimination of the aberrant clone or simply reregulation of the dysfunctional clone. Such studies will be important for our understanding of the physiologic and pathophysiologic mechanisms of chemopreventive response at the cellular level.

Retinoids

Suppression of Carcinogenesis by Retinoids. Retinoids have been shown to suppress carcinogenesis in a variety of animal models for cancer of the breast, lung, skin, prostate, bladder, oral cavity, and others (3). In the two-stage mouse skin carcinogenesis model, topically applied retinoids exhibited suppressive effects on tumor promotion but had no effect on the initiation step. In other models, retinoids administered in the diet were able to suppress tumor development even in an adjuvant setting after excision of the first tumor. The mechanisms underlying the anticarcinogenic activity of retinoids are not fully elucidated. They appear to be strongly associated with the pleiotropic effects that retinoids exert on the growth and differentiation of normal, premalignant, and malignant cells in vitro and in vivo that are described below.

Effects of Retinoids on Cell Growth and Apoptosis.

The growth and differentiation of various normal and malignant cells in culture is modulated by retinoids (21). Many tumor cell types, including melanoma, neuroblastoma, glioma, retinoblastoma, embryonal carcinoma, carcinomas of the lung, breast, prostate, bladder, colon, skin, head and neck, cervix, various types of sarcoma, and a variety of hematopoietic cancers (lymphomas, leukemias, myelomas, premonocytic leukemia, promyelocytic leukemia) often exhibit a decrease in growth rate after exposure to retinoids (21–23). Maintenance of the growth-inhibited stage usually requires the continuous presence of retinoids. Many tumor cells exhibit an anchorage-independent growth as spheroids or colonies in agar, which is considered to be a hallmark of malignant transformation in vitro. Treatment of various transformed and tumor cells with retinoids restores anchorage dependence and inhibits the ability of the cells to grow in suspension. Often, cells that are only marginally inhibited in monolayer culture show marked inhibition of anchorage-independent growth in agarose (21, 23). This assay has also been performed with cells dissociated from fresh tumor biopsies and has demonstrated a variable sensitivity of different tumor cells to different retinoids.

Retinoids also induce apoptosis in mesenchymal, neuroectodermal, hematopoietic, and epithelial cells during normal development and in cultured transformed and tumor cells (24, 25). Apoptosis induced by ATRA in neuroblastoma cells and in ATRA-resistant HL-60 myeloid leukemia cells was independent of differentiation induction. In contrast, ATRA enhanced apoptosis in HL-60 myeloid leukemia cells only subsequent to inducing cell differentiation into neutrophil-like cells. Although the mechanism of apoptosis induction by retinoids is still not fully understood, it may involve an increase in tissue transglutaminase expression and suppression of Bcl-2 expression. Indirect effects of retinoids on cell death may also be mediated via induction of transforming growth factor β that has been found to increase in cells undergoing apoptosis (26).

The effects of retinoids described above have important implications for chemoprevention of cancer. If retinoids can inhibit the growth of initiated cells or premalignant cells, then they should be able to suppress carcinogenesis by preventing the expansion of the precursors of the malignant lesions. Induction or enhancement of apoptosis of DNA-damaged cells, initiated cells, or premalignant cells could be a powerful mechanism for elimination or suppression of the aberrant clones of cells that otherwise would give rise to a malignant lesion.

Effects of Retinoids on Cell Differentiation. Naturally occurring retinoids act as physiological regulators of embryonal development and maintain the proper differentiation of many
tissues in the adult (27). Natural and synthetic retinoids administered at pharmacological doses can restore regulation of differentiation and growth in certain premalignant and malignant cells in vitro and in vivo. Retinoids also act pharmacologically to restore regulation of differentiation in certain malignant cells in vitro (23). The effects of retinoids on cell differentiation have been studied extensively, primarily in cultured cell lines derived from EC, normal and malignant keratinocytes, premonocytic and myeloid leukemias, neuroblastoma, and melanoma. In most of these cell types, retinoids enhance differentiation (23). Retinoids usually enhance predetermined programs in cells that have the potential to undergo differentiation along more specific pathways. For example, in F9 embryonal carcinoma cells, retinoids induce endodermal differentiation, whereas in human embryonal carcinoma cells, retinoids induce a neuronal differentiation. In P19 EC cells, retinoids can induce both myogenic differentiation and neuronal differentiation depending on the concentration of retinoid used. In HL-60 myeloid leukemia cells that have the potential to undergo either myeloid or monocytoid differentiation, retinoids can only induce the myeloid pathway. In contrast, ATRA was able to induce three different pathways (ectodermal, mesodermal, and endodermal) in a developmentally pluripotent germ cell tumor. However, in cultured keratinocytes and SCCs, retinoids inhibit squamous differentiation (28). Because some premalignant lesions in normally nonkeratinizing epithelial tissues undergo aberrant squamous differentiation, the effect of retinoids can be viewed as restoration of the normal nonkeratinizing phenotype (28).

Because of their ability to enhance the differentiation of malignant cells, retinoids have been front-runners in “differentiation therapy” trials aimed at inducing the differentiation of tumor cells in vivo (29, 30). Some of the concepts underlying this approach are also applicable for chemoprevention. For example, agents that can reverse the aberrant differentiation state of preneoplastic cells to a normal state may prevent carcinogenesis by suppressing the growth of the cells as a result of restoration of responses to normal growth controlling mechanisms.

A major development in the clinical application of retinoids was the discovery that ATRA can induce the differentiation of acute promyelocytic leukemia cells in vivo (31). There are only limited reports on induction of differentiation of solid tumor cells in vivo. A significant degree of differentiation was observed in teratocarcinomas growing in syngeneic mice after several retinoids were administered in the diet (32).

Pleiotropic Changes in Cell Phenotype Induced by Retinoids. Numerous reports described a variety of phenotypic changes in tumor cells, and many of those changes could play a role in either growth inhibition or differentiation induction (32). The ability of retinoids to modulate gene expression is the most plausible mechanism by which they can modulate the differentiation and growth of malignant cells or suppress the progression of premalignant cells to malignant lesions by redirecting their differentiation. The identity of the genes that control the expression of the premalignant or the malignant phenotype is not known; however, the restoration of normal differentiation by retinoids may represent a part of a retinoid-dependent program of gene expression that modulates cellular interactions and signal transduction. It is well established that cell-cell communication is disrupted at early stages of carcinogenesis. Therefore, the ability of retinoids to enhance gap junctional communication via increased expression of connexin 43 may be relevant for the chemopreventive activity of retinoids. The interactions of cells with the extracellular matrix by means of cell surface components may be important for expression of tissue-specific genes and for preventing conversion of intraepithelial neoplasia to invasive malignant carcinoma. Retinoids induce the expression of integrins as well as their cognate extracellular matrix components such as laminin, proteoglycans, hyaluronic acid, and collagen type IV in embryonal carcinoma cells, and some matrix proteins can also be induced in other cell types. Cell adhesion molecules that mediate cell-to-cell adhesion are also increased by retinoids.

Important processes of signal transduction are modulated by retinoids. Thus, retinoids can either increase or decrease the expression of a large number of cell surface receptors for a variety of cytokines and growth factors. Their effects are often cell-type specific and may reflect the cellular state of differentiation. A characteristic effect of retinoids is to subvert growth stimulatory signals mediated by different receptors. For example, RA has been reported to suppress the transcription of EGFR in cervical carcinoma cells and to decrease the level of the related HER2/neu on the surface of human breast carcinoma cells. Other targets for retinoid modulation are cytokines and growth factors. RA was found to decrease the level of TGF-α in human EC cells and thereby decrease their growth. Other suppressive effects of retinoids on growth factor expression include the decreased level of insulin-like growth factor-I mRNA in glioma cells. Induction of the TGF-β1 receptor expression and TGF-β1 protein production in HL-60 cells by RA has been shown to generate an inhibitory autocrine loop. Retinoids were also reported to modulate the expression of various proto-oncogenes, suppressor genes, and cell cycle regulatory kinases that are either reflecting the induction of a more differentiated phenotype or the reduced growth rate of the cells (23, 26, 32).

In addition to the effect of retinoids on cell surface-mediated signal reception, they also intervene with intracellular signaling via second messengers (32, 33). Both increases and decreases were reported in the level of pKs A and C. Neuronal differentiation of human EC cells was accompanied by increases in both pKA and pKC, and an increase in pKC-α was implicated in the induction of melanoma cell differentiation. In contrast, neuronal differentiation of neuroblastoma cells was accompanied by a decreased level of pKC-α. These results suggest that many of the effects of RA are cell-type specific and cannot be generalized. It is still not clear how retinoids alter the expression of the above genes. Some of them may be regulated directly, whereas others may be altered indirectly as a result of the initiation of a new gene expression program or in response to an altered growth state.

Clinical Aspects

Chemoprevention is not yet considered standard clinical practice. However, retinoids (13cRA, retinyl palmitate, fenretinode) appear to be effective in reversing premalignant oral lesions and preventing second primary tumors in cancers of the lung and upper aerodigestive tract. Several positive trials have raised
significant enthusiasm for cancer chemoprevention (Table 1), and many ongoing randomized trials will provide more definitive information (3).

**Head and Neck.** Five randomized trials of either natural or synthetic retinoids have been conducted in oral premalignancy (33–37). Conducted in 1986, the first was a 3-month placebo controlled trial of 13cRA (2 mg/kg/day) in 44 evaluable patients (33). The complete plus partial response rate was 67% in the retinoid arm and 10% in the placebo arm (P = 0.0002). The histological improvement rate (reversal of cytological abnormalities) also was significantly better in the retinoid than in the placebo arm (54% versus 10%, P = 0.01). This high-dose, short-term regimen created two major problems, however. First, the regimen had substantial, unacceptable toxicity for this low-risk setting. Second, over 50% of responders recurred or developed new lesions within 2–3 months of stopping treatment.

A follow-up randomized trial of a maintenance nature was designed to solve the drawbacks of the earlier trial (34). Patients received a 3-month induction course of high-dose 13cRA (1.5 mg/kg/day) followed by a 9-month maintenance regimen of either low-dose 13cRA (0.5 mg/kg/day) or β-carotene (30 mg/day) in patients with responding or stable lesions after induction. Responses in the induction phase was 55%. In the second phase, 8% of the retinoid patients progressed versus 55% of β-carotene patients (P < 0.001). Retinoid maintenance therapy was well tolerated; no patients discontinued therapy because of toxicity.

Following these two trials, three other randomized retinoid studies were conducted in oral premalignancy. All three trials, two of an induction (35, 36) and one of a maintenance (37) nature, achieved significant retinoid chemopreventive activity.

Extensive randomized study of retinoids also has occurred or is ongoing in the setting of second primary tumors prevention. Hong et al. (38) achieved a significant reduction in the rate of SPTs in a randomized adjuvant trial of high-dose 13cRA in 103 head and neck cancer patients. Following definitive local therapy of stage I–IV primary cancer, patients were assigned randomly to receive either placebo or 13cRA (50–100 mg/m²/day) for 12 months. At 32-month median follow-up, no significant differences in primary disease recurrence (local, regional, or distant) or survival existed between the two groups. The SPT rate, however, was significantly lower in the retinoid than in the placebo arm. SPTs had developed in only 4% of the 13cRA-treated patients compared with 24% of placebo patients (P = 0.005). The vast majority of SPTs occurred in carcinogen-exposed fields of the aerodigestive tract within the head and neck, lungs, and esophagus. Substantial side effects occurred, including dry skin, cheilitis, conjunctivitis, and hypertriglyceridemia. Only one-third of retinoid-treated patients completed the full 12-month intervention.

An updated analysis of Hong’s (38) adjuvant trial was conducted after a 55-month median follow-up (versus earlier 32 months; Ref. 39). Although the gap in total SPTs (located in all sites) between the two arms narrowed substantially, the retinoid group continued to have fewer than did the placebo group (14% versus 31%, P = 0.04). The chemopreventive effect on tobacco-related SPTs (located in the aerodigestive tract) persisted at nearly the same strength, however: SPTs developed in only 7% of the retinoid group versus 33% of the placebo group (P = 0.008). This long-term benefit occurred despite the relatively short-term intervention and dose reductions required by toxicity in approximately one-third of the patients.

Based on positive SPT results and toxicity problems of this high-dose adjuvant trial (38, 39) and on positive results of the recently completed maintenance (low-dose) trial of 13cRA in oral premalignancy (34), a multicenter Phase III trial of low-dose 13cRA to prevent SPTs associated with stage I and II head and neck cancer was initiated. A recent report of this ongoing trial indicates that the low-dose 13cRA is being well tolerated (40).

A recent French trial assessed the synthetic retinoid etretinate in preventing SPTs following definitive therapy of early-stage squamous cell carcinomas of the oral cavity and oral pharynx (41). Etretinate or placebo was randomly given at doses of 50 mg/day for 1 month followed by 25 mg/day for 2 years. The study arms did not differ significantly in respect to SPT rates. Although interpretation of this trial is hampered by the lack of details on study compliance and SPT diagnostic criteria, certain valuable data were reported. This trial confirmed other prospective data on the high rate of head and neck cancer-associated SPTs (3, 42–44): 24% of the placebo patients developed SPTs (evaluated after 41-month median follow-up). Also, approximately 80% of the SPTs developed in the head and neck, lungs, or esophagus (41), which is consistent with the field carcinogenesis theory behind aerodigestive tract cancers.

**Lung.** An uncontrolled French trial of etretinate (25 mg/day for 6 months) demonstrated a decline in the extent of

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**Table 1 Completed randomized retinoid chemoprevention trials**

<table>
<thead>
<tr>
<th>Study</th>
<th>Setting</th>
<th>No. of patients</th>
<th>Agent (dose)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head and neck</td>
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<td></td>
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</tr>
<tr>
<td>Hong et al. (33)</td>
<td>Oral leukoplakia</td>
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<td>Isotretinoin (2 mg/kg/day)</td>
<td>Positive</td>
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<td>Stich et al. (35)</td>
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<td>Vitamin A (200,000 IU/wk)</td>
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<td>Retinamide (40 mg/day)</td>
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<td>70</td>
<td>Isotretinoin (0.5 mg/kg/day)</td>
<td>Positive</td>
</tr>
<tr>
<td>Costa et al. (37)</td>
<td>Oral leukoplakia</td>
<td>153</td>
<td>Fenretinide (200 mg/day)</td>
<td>Positive</td>
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<tr>
<td>Hong and colleagues (38, 39)</td>
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<td>103</td>
<td>Isotretinoin (100–50 mg/m²/day)</td>
<td>Positive</td>
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<tr>
<td>Bolla et al. (41)</td>
<td>Prior cancer</td>
<td>316</td>
<td>Etretinate (50–25 mg/day)</td>
<td>Negative</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
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<tr>
<td>Arnold et al. (46)</td>
<td>Metaplasia (sputum)</td>
<td>150</td>
<td>Etretinate (25 mg/day)</td>
<td>Negative</td>
</tr>
<tr>
<td>Lee et al. (47)</td>
<td>Metaplasia (biopsy)</td>
<td>87</td>
<td>Isotretinoin (1 mg/kg/day)</td>
<td>Negative</td>
</tr>
<tr>
<td>Pastorino et al. (48)</td>
<td>Prior NSCLC</td>
<td>307</td>
<td>Retinyl palmitate (300,000 IU/day)</td>
<td>Positive</td>
</tr>
</tbody>
</table>
squamous metaplasia in the bronchial epithelium of chronic smokers (45). This study led to two randomized trials in similar settings. The first (conducted in Canada) evaluated the ability of etretinate (25 mg/day) to reverse sputum atypia in chronic smokers (46). No difference in degree of atypia occurred between the etretinate and placebo groups after 6 months of treatment. The second randomized trial (conducted in the United States) assessed the efficacy of 13cRA in reversing bronchial metaplasia or dysplasia in chronic smokers (47). Metaplasia regressed in approximately 50% of the subjects of both the retinoid and placebo arms. Only smoking cessation was associated significantly with a reduction in metaplasia index after the 6-month intervention. Although negative in comparison to the placebo, the U.S. and Canadian retinoid results were similar to the etretinate results in the earlier uncontrolled French trial. These findings underscore the critical importance of placebo-controlled designs to establish drug activity in chemoprevention trials using intermediate end points, such as reversal of premalignant conditions.

Retinoids also have been treated in the setting of preventing SPTs associated with lung cancer. Pastorino et al. (48) divided 307 patients who had been resected for stage I non-small cell lung cancer into one group receiving retinyl palmitate (300,000 IU/day) and another group with no intervention for 1 year. Eighteen patients in the retinyl palmitate arm and 29 patients in the control group developed SPTs. The retinoid’s effect appeared to be most pronounced in reducing tobacco-associated SPTs. After a median follow-up of 46 months, tobacco-related SPTs developed in 13 retinyl palmitate-treated patients and in 25 control patients. The time to development of tobacco-associated SPTs also was favorable (increased) in the retinyl palmitate arm (P = 0.045). Greater than 80% adherence indicated that the high-dose retinyl palmitate was well tolerated. Only three patients dropped out of the retinoid arm because of toxicity.

The encouraging results of Pastorino et al. (48) and retinoid activity in related carcinogenic systems led to the design of several Phase III retinoid trials in the setting of SPT prevention. Two very important such trials are currently underway in Europe and the United States (3, 43, 44). The European trial, called Euroscan, is a multicenter trial with a randomized 2 × 2 factorial design involving retinyl palmitate and N-acetylcysteine to prevent SPTs following definitive therapy of early-stage head and neck cancer. The U.S. trial also is a large multicenter trial, which involves low-dose 13cRA to prevent SPTs following complete resection of stage I non-small cell lung cancer (Intergroup NCI I 91-0001).

Translational Research

Suppression of RAR-β Expression in Oral Premalignant Lesions and Induction with 13cRA. The ability of retinoids to regulate gene expression is the most plausible mechanism by which they can modulate the differentiation and growth of malignant cells or suppress the progression of premalignant cells to malignant lesions by redirecting their differentiation. To modulate gene expression, retinoids must transmit signals to the cell nucleus. The mechanism of this signal transduction is beginning to be unraveled as the understanding of the roles of the major components of the pathway increases. Nuclear retinoid receptors appear to play important roles in the series of events that is initiated with the uptake of a retinoid by a target cell and culminates in the modulation of gene transcription in the cell’s nucleus (Fig. 1).

The nuclear retinoid receptors are members of the steroid/thyroid hormone/vitamin D receptor family (49). They act as ligand-activated trans-acting transcription factors and mediate the effects of retinoids on gene expression, thereby altering the growth and differentiation of normal and tumor cells (21, 22). There are two types of retinoid nuclear receptors: RARs and RXRs. Each of the receptor types has at least three subtypes: α, β, and γ. RAR-α, RAR-β, and RAR-γ were localized on chromosomes 17q21.1, 3p24, and 12q13, respectively, and RXR-α, RXR-β, and RXR-γ were localized on chromosomes 9q34.3, 6p21.3, and 1q22–23, respectively (22, 23, 49–52). The RARs bind ATRA and 9-cis RA, whereas the RXRs bind 9-cis RA but not ATRA. The RXRs and RARs form heterodimers before binding to specific DNA sequences characterized by direct repeats of (A/G)GGTCA separated by two or five nucleotides, although complex elements have also been identified (22, 23, 49).

Physiological retinoids may play an important role in the prevention of cancer development by maintaining proper differentiation of hematopoietic and epithelial tissues. Because the retinoid receptors appear to be the proximate mediators of retinoid action on gene expression, abrogation of their expression or function could result in cancer development. Studies with head and neck squamous cell carcinomas (53) and lung carcinomas (54) have failed to detect RAR-β in a considerable number of cell lines, and proposed that RAR-β reduction may be linked to cancer development. The ability of transfect d RAR-β to suppress the tumorigenicity of a human lung carcinoma cell line provided further support for this hypothesis. We explored this hypothesis in the context of upper aerodigestive
A B  
A B

Fig. 2 Detection of RAR-β mRNA in adjacent normal tissue and in hyperplastic, dysplastic, and malignant tissues present in the same histological section of a head and neck squamous cell carcinoma surgical specimen using antisense RAR-β riboprobe. A, adjacent normal tissue; B, hyperplastic tissue; C, dysplastic tissue; and D, carcinoma. × 150.

tract carcinogenesis in vivo. Digoxigenin-labeled antisense riboprobes RARs and RXRs were used for in situ hybridization to histological sections of specimens from 7 normal volunteers and 31 head and neck SCC patients (55). All 31 tissue specimens contained carcinomas. Sixteen also contained dysplastic lesions, 22 also contained hyperplastic lesions, 17 also contained adjacent normal tissue, and 6 contained all four types of tissue. All specimens from normal volunteers expressed RAR-α, RAR-β, and RAR-γ, and RXR-α. Similar levels of RAR-γ mRNA were detected in about 94% of the adjacent normal, hyperplastic, dysplastic, and malignant tissues, whereas RAR-α mRNA was detected in 94% of the adjacent normal tissues and hyperplastic tissues and in 87% of the dysplastic tissues and 77% of the SCCs. In contrast, RAR-β mRNA was detected in about 70% of the adjacent normal and hyperplastic lesions, and its expression decreased further to 56% of the dysplastic lesions and 35% of the SCCs. An example of the gradual decrease in RAR-β expression during head and neck cancer development is shown in Fig. 2. To determine whether the decrease in RAR-β expression could be detected in patients with oral premalignant lesions without cancer, we analyzed histological sections from 52 patients with leukoplakia and biopsies from the oral cavity of 7 normal volunteers without premalignant lesions. RAR-α, -β, and -γ and RXR-α were expressed in all normal specimens. In contrast, RAR-β was detected in only 40% of the oral leukoplakia specimens, while RAR-α and RAR-γ were expressed in 88 and 94% of the specimens, respectively. After a 3-month 13-cRA treatment of 1.5 mg/kg/day, a significant increase in the percentage of leukoplakias expressing RAR-β rose from 40 to 90% of the cases. Furthermore, the majority of oral premalignant lesions that responded clinically to 13cRA treatment showed an increase in RAR-β level (56).

A similar analysis of specimens from normal lung, bronchial metaplasia, lung tissue from resection margin, dysplastic tissue, and lung cancer demonstrated that RAR-β was not detected in 50% of the dysplastic tissues and >50% of lung adenocarcinoma and SCCs, whereas the expression of the other receptors was not altered. The decrease in RAR-β expression was observed in histologically normal bronchial epithelium at the resection margin (57). These results indicate that RAR-β may be a useful intermediate marker in head and neck and lung carcinogenesis prevention studies because its expression is decreased in early stages of carcinogenesis, and its expression is induced in vivo during treatment with 13cRA, and this increase in vivo is associated with response to the preventive agent.

p53 and Retinoid Response. A strong rationale supports translational studies of p53 within head and neck cancer chemoprevention trials. Alterations of the p53 gene and its protein products occur frequently in normal-appearing and premalignant tissue adjacent to head and neck tumors. Shin et al. (58) detected p53 protein accumulation in 21% of the histologically normal specimens, 29% of the hyperplastic specimens, 45% of the dysplastic specimens adjacent to cancer, and 45% of the invasive cancer specimens. The study of Shin et al. (58) also showed that p53 protein overexpression becomes progressively more abnormal (e.g., increased p53 expression in suprabasal epithelium) in association with histological progression. Direct molecular studies also have revealed carcinogenesis-related abnormalities of p53. Boyle et al. (59) observed p53 inactivation in 43% (28/65) of the invasive cancers they studied. Other data suggest that retinoids can modulate p53 mRNA and p53 protein levels associated with carcinogenesis in certain in vitro systems (60).

All of these p53 findings led to a prospective study of p53 in the retinoid-oral premalignancy model (61). Cancer-free patients with advanced oral premalignant lesions were assessed for p53 protein accumulation. This assessment was aided by a very sensitive microwave technique (previously shown to enhance epitope retrieval and, therefore, p53 detection). Widely ranging levels of p53 protein were detected in 89% of the 45 lesions but not in any of 8 oral cavity specimens from 7 healthy nonsmoking controls. Levels of p53 protein accumulation increased in association with histological progression [paralleling findings in the lung and esophagus (62, 63)]. The pattern of p53 expression also was related to histological grade; increased p53 expression in the parabasal layer occurred in direct association with increasing histological severity.

Other findings of this study were a lack of p53 modulation by 13cRA and a significant correlation between levels of p53
accumulation and lesion resistance to 13cRA, possibly related to a lack of RAR-β up-regulation. The mechanism of this resistance is unclear. Retinoid resistance may be due in part to altered p53 function and/or other associated genetic alterations. Alternatively, p53 protein overaccumulation may mark a stage of oral carcinogenesis that has advanced beyond retinoid chemoprevention’s reach and requires entirely different interventions, such as gene therapy (64). Future multidisciplinary studies will be required to explain the complex relationship connecting oral carcinogenesis, p53 gene, and protein expression and retinoid chemoprevention.

Future Directions
Over the last decade, strategies for the control of epithelial cancers have shifted from treatment of metastatic disease to prevention, and chemoprevention is a very promising new strategy for reducing the morbidity and mortality of the disease.

The most important issue is the definitive end point of chemoprevention trials, especially in Phase III trials, which is cancer incidence. This end point usually needs a huge sample size and lengthy time to determine the efficacy of the agent. Biological markers that may serve as potential intermediate end points should be comprehensively investigated through translational research. Validated intermediate end points could reduce the trial population, expense, and duration of Phase III trials.

Retinoids appear to be most effective in intervening with the carcinogenic process in the aerodigestive tract. Intensive translational investigation is underway to increase our understanding of the mechanisms of retinoid reversal of premalignant lesions at the cellular and molecular levels. Synthetic retinoids, which exhibit specific binding to individual nuclear receptors, and retinoids that are potent inducers of apoptosis may be more effective in cancer prevention than currently available ones.

References
Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic.

W Ki Hong, S M Lippman, W N Hittelman, et al.


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