Dietary retinoids and carotenoids in rodent models of mammary tumorigenesis

Richard C. Moon and Andreas I. Constantinou
University of Illinois at Chicago, College of Medicine, Department of Surgical Oncology, Chicago, IL 60612, USA

Key words: breast cancer, carcinogenesis, carotenoids, chemoprevention, differentiation, in vivo, retinoids

Summary

In this review of the scientific literature the relationship between retinoids, carotenoids, and mammary carcinogenesis is examined. Several retinoids have shown promise as chemopreventive agents against chemically induced mammary carcinogenesis in mice and especially in rats. The most promising retinoids are retinyl acetate (RA) and N-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide). In rats, dietary administration of these retinoids reduced tumor incidence and multiplicity, and increased the latency of DMBA or MNU-induced mammary cancers. In mice, 4-HPR reduced the number of hyperplastic alveolar nodules and the number of tumors in MTV- and MTV+ mice, respectively. Among retinoids, 4-HPR is at present the most promising analogue, due to its ability to concentrate in the mammary gland. The combination of 4-HPR with tamoxifen not only is more effective in suppressing breast cancer than either agent alone, but also inhibits the appearance of subsequent cancers following the surgical removal of the first tumor. These studies suggest that retinoids, like tamoxifen, may be applicable to the prevention of contralateral breast cancer in women who underwent breast cancer surgery. It is also becoming evident that differentiation therapy and chemoprevention can become attractive alternative approaches to intensive cytotoxic chemotherapy. The role of carotenoids in the prevention of mammary carcinogenesis, however, is ambiguous. Poor absorption and low levels of carotenoids that reach the target tissues complicate interpretation of data in rodent models of mammary carcinogenesis. Very few animal studies are presently available in which purified carotenoids were found effective against mammary carcinogenesis. These results do not justify undertaking clinical evaluation of individual carotenoids against breast cancer at this time.

Introduction

The inhibition of chemically induced tumors in laboratory animals by natural compounds such as isothiocyanates and indoles, which are found in fruits and vegetables, was studied originally by W. Waterberg and his associates [1]. This work gave rise to the concept of chemoprophylaxis of carcinogenesis. The term “cancer chemoprevention, however was suggested originally by M.

Address for correspondence and offprints: Andreas I. Constantinou, Ph.D., University of Illinois at Chicago, College of Medicine, Department of Surgical Oncology (MC 820), 840 South Wood Street, Chicago, IL 60612; Tel: (312) 413-1155; Fax: (312) 996-9365; e-mail: andreasc@uic.edu
Sporn in a series of hallmark studies performed originally using retinoids [2]. Cancer chemoprevention is defined as the prevention of cancer in human population by ingestion of chemical agents that prevent carcinogenesis. These agents may prevent initiation of transformation or delay the progression of transformed premalignant cells to malignant disease [3]. To achieve this preventive role, a chemopreventive agent should enhance the physiological processes that protect an organism against the growth of abnormal cells with a potential of developing into an invasive cancer. Since retinoids are involved in the maintenance and regulation of differentiation and cancer is a process in which loss of differentiation occurs, retinoids have been targeted as potential cancer chemopreventive agents.

The role of retinoids as chemopreventive agents has been examined in numerous in vivo and in vitro tumor model systems [4]. Such studies have shown that retinoids can reverse premalignant changes in the epithelium of mouse prostate glands in organ culture [5] and inhibit malignant transformation of cells in vitro, irrespective of whether the transformation is induced by ionizing radiation [6], chemical carcinogens [7], or transforming polypeptides [8].

The potential use of carotenoids such as beta-carotene for the chemoprevention of cancer in humans would appear to be of great significance. Beta-carotene is essentially non-toxic in humans, and a significant body of epidemiologic evidence supports an inverse association between intake of foods high in carotenoids and the risk of cancer development in a number of target organs. However, the poor absorption of carotenoids in rodents has limited the value of the tumor model in assessing the effect of the chemopreventive agents against mammary carcinogenesis. Thus, the epidemiologic data suggesting a protective effect conferred by dietary carotenoids have been difficult to confirm in an experimental model.

The literature on the effects of retinoids and carotenoids as chemopreventive agents in a variety of animal models of experimental carcinogenesis has been reviewed previously [9]. The present review is directed exclusively on the effects of the two classes of chemopreventive agents on rodent mammary tumorigenesis.

**Retinoids**

The general group of retinoids includes some of the most promising natural and synthetic chemopreventive agents. Chemically, the retinoids are defined as diterpenoids derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion. However, this definition does not account for more potent, newer synthetic retinoids such as tricyclic or tetracyclic retinoidal benzoic acid derivatives, which may not be diterpenoids or may not be derived from the monocyclic parent compound. Structures of certain retinoids derived from monocyclic structures, such as retinol or retinoic acid, as well as multicyclic structures such as retinoidal benzoic acid, can be modified to yield almost an unlimited number of retinoids. More than 1000 retinoids have been synthesized, and many have been screened for biological activity by use of the tracheal organ culture assay described by Clamon et al [10].

Although numerous retinoid analogs have been evaluated for their potential usefulness in preventing carcinogenesis, new retinoids with less than classical chemical structure are continually being synthesized and studied. These third-generation retinoids include arotinoids, benzoylaminobenzoic acids, stilbene carboxylic acids, stilbenoids, and conjugated retinoids. Retinoids selected for optimal efficacy in animal studies of experimental carcinogenesis have been previously evaluated with in vitro procedures. These include: a) induction of terminal differentiation of transformed cells associated with loss of neoplastic character [11-15], b) hamster tracheal organ culture introduced by Sporn and his colleagues [16], c) mouse prostate organ culture [5], and d) mouse mammary gland organ culture [17,18].

Data gathered from studies of the chemopre-
vention of carcinogenesis strongly suggest that retinoids have a moderate degree of target-organ as well as species specificity. Several examples can be cited to illustrate this phenomenon. 13-cis-retinoic acid (13cRA) is one of the most effective retinoids in preventing chemically-induced carcinogenesis in the urinary bladder of mice and rats, as well as in preventing two-stage carcinogenesis in the skin. However, it is ineffective against DMBA- or MNU-induced mammary carcinogenesis. Similarly, 4-HPR is highly effective against mammary and urinary bladder carcinogenesis, but is ineffective against carcinogenesis in the esophagus or colon. Retinyl methyl ether, which is effective against DMBA-induced mammary carcinogenesis, has little inhibitory effect against MNU-induced mammary carcinogenesis in rats. Retinyl acetate is stimulatory or without effect in virally or hormonally induced mammary carcinogenesis in mice. Finally, several retinoids, such as trimethylmethoxyphenyl (TMMP) analogues, benzoic acid derivatives, and stilbenoids are very active in two-stage carcinogenesis in the skin but inactive against mammary carcinogenesis [19].

Collectively, the results of these studies suggest that retinoid action may be tissue-dependent, and may result from differences in the tissue distribution and metabolism of retinoids among the various organs. Most of the retinoids that have been studied for chemopreventive effects in in vivo carcinogenesis experiments were selected from the list of retinoids found effective in the tracheal organ-culture assay. However, a consistent correlation is not often observed between tracheal organ culture and chemopreventive efficacy in vivo. Thus, it may be necessary to use tissue-specific culture systems in order to predict the responsiveness to a particular retinoid in vivo.

Animal models of mammary carcinogenesis

A number of tumor models for various target organs are available to study modulation of the carcinogenic process by exogenous factors. Animal models of mammary carcinogenesis should satisfy the following criteria: (a) development of mammary cancer should be relatively rapid, (b) cancer should develop only in the mammary tissue, (c) cancer should mimic growth characteristics of human breast cancer, (d) the chemical carcinogen should cause little or no systemic toxicity, and (e) histogenesis and histopathology of the tumors should be similar to that of the human counterpart. Among the existing models for experimental breast carcinogenesis studies are those utilizing either DMBA or MNU as the carcinogens.

Both of these models are highly reproducible and have been extensively utilized in chemoprevention experiments. The MNU-induced tumor model was developed by Gullino et al [20] and subsequently modified in our laboratory [21]. In the MNU model, female Sprague-Dawley rats at about 50 days of age are injected with the carcinogen intravenously with 50 mg/Kg of body weight. In the DMBA model, female Sprague-Dawley rats, at age 50 days, are usually given a single intragastric injection of 12 mg of the carcinogen. Since DMBA is activated in the liver the DMBA model is useful in identifying agents which inhibit carcinogen activation such as those inhibiting cytochrome P-450. DMBA-induced tumors are mainly adenocarcinomas and fibroadenomas which are encapsulated and non-invasive. MNU is a direct-acting carcinogen and it produces predominantly adenocarcinomas, which, like human breast tumors, are invasive. Thus, the MNU model of rat mammary carcinogenesis is a better model for human breast cancer. Both carcinogens produce tumors that, like human breast cancers, are hormone dependent. In our laboratory, carcinogen-treated controls (DMBA or MNU), typically show 90-100% tumor incidence, while the multiplicity ranges from 3-7 tumors per animal with an average tumor latency of 65-80 days. The efficacy of a chemopreventive agent is measured as percent reduction in adenocarcinoma incidence or multiplicity, or increase in latency of tumor appearance, when compared to the carcino-
gen-treated controls. The rat model has been the most systematically used for assessing the effects of retinoids on chemically induced mammary tumors.

Although the sequence of events and dose-response relationships for the induction of mammary tumors are well defined in the above rat tumor models, mouse models of mammary carcinogenesis are less well characterized, and recent studies using the mouse model have been inconsistent. Moreover, most of the studies involving mice have utilized either virally-induced or hormonally-induced mammary tumors in C3H or GR mice, respectively.

**Effects of retinoids on mammary carcinogenesis**

In most animal studies, retinoids inhibit the progression phase of mammary carcinogenesis. Some of the most convincing evidence for the chemoprevention of cancer by retinoids comes from studies of chemical carcinogenesis in the mammary gland in rats. The criteria used for determining chemopreventive efficacy in these models are: a) an increase in the latency of first tumor appearance, b) a decrease in the number of cancers per animal (tumor multiplicity), and c) a decrease in the number of animals with cancer (tumor incidence). The earliest study to report an inhibition of mammary carcinogenesis was that of Moon et al [22], who found a 52% reduction in the incidence of mammary cancer in rats treated with DMBA and 2.5 mg of retinyl acetate per day as compared to the incidence in animals receiving DMBA and a placebo diet. A previous report by Schmahl et al [23] had indicated that retinyl palmitate had no influence on DMBA-induced mammary tumorigenesis. The discrepancy between these two studies was mainly attributed to the mode of administration of the retinoids. Schmahl et al administered the retinoid once a week by gastric intubation, whereas Moon et al. administered it in the diet, thereby ensuring a more sustained exposure of the target cells to the retinoid. Subsequent studies by Grubbs et al. [24] confirmed the suppressive effects of retinyl acetate on DMBA-induced mammary carcinogenesis and also identified retinyl methyl ether as more effective than retinyl acetate in preventing the appearance of mammary cancer. In these studies, feeding of the retinoids began seven days after DMBA treatment, a time at which the uptake and binding of the carcinogen by mammary parenchymal cells was essentially complete [25]; the results indicate that the retinoids were inhibiting the progression rather than the initiation of mammary tumorigenesis. In addition to demonstrating the effect of retinyl acetate on DMBA-induced mammary tumorigenesis, Moon et al [26] have also shown that retinyl acetate inhibits mammary carcinogenesis induced by the intravenous injection of the direct-acting carcinogen MNU. Both the incidence of mammary cancer and the tumor number were diminished at all doses of this carcinogen, and those animals receiving the low dose of carcinogen and retinyl acetate did not develop tumors.

Since the original studies by Moon and colleagues, a series of synthetic retinoids have been evaluated for efficacy against chemically induced mammary cancer. Of all the retinoids evaluated against mammary carcinogenesis, 4-HPR and retinyl acetate appear to be the most efficacious. However, in contrast to 4-HPR, retinyl acetate accumulates to a large extent in the liver in the form of retinyl esters, causing significant hepatotoxicity [27]. By comparison, 4-HPR accumulates in the mammary gland in a dose-related manner [28] and is metabolized by the mammary epithelial cells in both rodents [29] and humans [30]. 13-cis-retinoic acid has little effect upon the appearance of MNU-induced mammary carcinomas; retinyl methyl ether is of intermediate efficacy, although the latter compound is extremely effective against DMBA-induced mammary carcinogenesis. Thus, it is readily apparent that minor alterations in the basic retinoid structure can significantly alter the activity of the molecule with respect to the inhibition of chemical carcinogenesis of the mammary gland.
Seeking a better retinoid for the chemoprevention of mammary carcinogenesis, Hartmann and Bollag [31] have investigated the effectiveness of three arinoids against DMBA-induced mammary carcinogenesis. The arinoids evaluated were Ro 15-0778, a non-polar arinoid; Ro 15-1570, an arinoid ethyl sulfone; and Ro 13-6298, an arinoid ethyl ester. Oral administration of these arinoids to DMBA-treated rats significantly inhibited the multiplicity and size of induced mammary tumors. The efficacy of Ro 15-0778 was confirmed in our laboratory [32]. It was observed that the dietary administration of 30 or 90 mg of this arinoid per kilogram of diet suppressed the multiplicity of mammary tumors in rats. Although neurotoxicity was noted at higher dietary concentrations of this arinoid, no adverse effects were observed at the chemopreventive concentrations used.

In studies in mice, it was found that RA did not influence (neither inhibited nor enhanced) tumor incidence, latency, or tumor number in C3H-AVY female mice positive for MTV [33]. However, using C3H mice negative for the MTV, it was found that the number of hyperplastic alveolar nodules (putative precancerous lesions) developing in animals receiving dietary 4-HPR was significantly less than that of control mice receiving the placebo diet, while RA did not affect nodulogenesis in these experiments. Welsch et al [34], on the other hand, have reported an enhancement of tumor development in nulliparous and multiparous mice of the GR strain fed a diet supplemented with RA, but inhibition with 4-HPR in C3H mice. Although the evidence supporting the chemopreventive activity of arinoids in models of rat mammary carcinogenesis is substantial, only a few studies describing the use of arinoids in mouse models of mammary carcinogenesis have been reported.

Recently, Grubbs et al [35] evaluated the effects of 4-HPR and retinyl acetate on the initiation and promotion of both DMBA- and MNU-induced mammary carcinogenesis in rats. Dietary retinyl acetate (1 mmol/kg diet) or 4-HPR (2 mmol/kg diet) was given to female Sprague Dawley rats two months before the administration of the two carcinogens. In the MNU model, they found a trend towards an increase in the number of cancers resulting from pretreatment with both arinoids. However, continued treatment during the initiation and promotion phases suppressed the multiplicity of cancers. In the DMBA-treated rats, pretreatment with arinoids increased the incidence of benign tumors without affecting the development of adenocarcinomas.

In the majority of chemoprevention studies, retinoid treatment was begun shortly after carcinogen administration. However, for clinical trials it is beneficial to know whether the initiation of retinoid treatment can be delayed for some period after the carcinogenic insult without a loss of chemopreventive activity. McCormick and Moon [36] conducted a detailed study in which mammary tumors were induced with two dose levels (25 mg/kg and 50 mg/kg of body weight) of MNU. Dietary administration of retinyl acetate (1 mmol/kg) was begun at 1, 4, or 8 weeks after carcinogen treatment for the high dose and at 1, 4, 8, 12, 16, and 20 weeks after carcinogen for the low dose of MNU. It was found that at the low dose of carcinogen, treatment with retinyl acetate could be delayed for up to 12 weeks without a loss of chemopreventive activity, whereas delaying the retinoid treatment for 20 weeks resulted in little protection against cancer development. On the other hand, at the higher concentration of MNU, retinoid treatment could be delayed only for up to 4 weeks without a loss in preventive activity. These results suggest that during the process of mammary carcinogenesis, there is a critical phase or point at which the cells are responsive to retinoid inhibition, and that after this time, retinoid treatment becomes less effective. It is also possible that after a certain period of time, both retinoid-resistant and retinoid-sensitive cells may be present in the lesion. Thus, any effect of the retinoid treatment on the sensitive cells may be obscured by replication of the resistant cells, unless critical measurements are made of tumor size with respect to time.
Retinoids suppressing tumor recurrence

For retinoids to be clinically acceptable for women at high risk for breast cancer, they must either have chemotherapeutic activity, suppress tumor recurrence after the surgical removal of an existing cancer, or prevent the development of additional tumors. The efficacy of retinoids as protective agents against the recurrence of mammary tumors or the development of subsequent tumors has been evaluated in experimental animals with retinyl acetate [36] and 4-HPR [37]. In these studies, animals were treated with the carcinogen and allowed to consume a control diet. When the first palpable tumor reached 1 cm in size, it was surgically removed and the animal was assigned to one of the treatment groups. During the first 2 months after tumor excision, very little quantitative tumor inhibition by the retinoid was evident, but there was a striking reduction in the appearance of subsequent tumors in the retinyl acetate-treated rats [38]. These results suggested that the inhibition of mammary cancer by the retinoid was due to suppression of the progression of early lesions.

In an other study [37], rats were treated with 35 mg/kg of MNU at 50 days of age and the first palpable tumor was surgically removed when it reached 0.5 cm in diameter. The animals were placed on a diet containing 4-HPR at 1, 2, or 3 mmol/kg. A placebo diet without 4-HPR served as a control. The retinoid decreased the development of new tumors and tumor multiplicity in a dose-related manner. The plasma level of 4-HPR increased with increasing dietary dose levels and was directly correlated with an increased survival of tumor-bearing animals. These studies clearly demonstrate the possibility of utilizing retinoids after the surgical removal of a tumor to prevent the occurrence of new tumors, and suggest that retinoids would also be applicable to the prevention of contralateral breast cancer in women at high risk. As a result of these studies 4-HPR is considered to be the most effective retinoid for the chemoprevention of breast cancer, and is now being evaluated clinically in Milan [39,40].

Combination chemoprevention studies with retinoids

Several investigators have demonstrated an interaction between retinoids and other modifiers of mammary carcinogenesis. In most cases, combined treatment affords greater protection against mammary carcinogenesis than either treatment alone. Carcinogen-induced rat mammary cancer models are subject to inhibition by both retinoids and modification of host hormonal status. Thus, ovarian hormone-dependent tumors regress following ovariectomy of the tumor-bearing animal, whereas in animals ovariectomized shortly after carcinogen administration, only ovarian hormone-independent tumors appear, and cancer incidence is low. The combination of ovariectomy and RA results in a synergistic inhibition of tumor incidence and multiplicity [41]. Similar results are obtained with 4-HPR. In a more recent study [42], it has been shown that tamoxifen and 4-HPR, when used in combination, are much more effective in inhibiting the development of new tumors than either agent alone.

Although hormonal modification of experimental mammary tumorigenesis is well established, the evidence cited above indicates that the retinoids also effectively alter mammary tumorigenesis. These data suggest the existence of populations of preneoplastic and/or neoplastic cells displaying differential sensitivity to the retinoids and hormones. Whether retinoids preferentially suppress the growth of hormone-dependent cell populations, reverse the neoplastic potential of these cells, or induce terminal differentiation of preneoplastic cells is presently unknown.

Carotenoids

Using mammary organ culture, Som et al [43] reported that mammary epithelial cells were transformed 24 h after treatment with DMBA. The transformation process was associated with appearance of nodule-like alveolar lesions, which
are neoplastic. Beta-carotene inhibited DMBA-induced transformation of the mammary glands in vitro, acting both at the initiation and the promotional stages. This study initiated further testing of carotenoids as potential chemopreventive agents against mammary carcinogenesis in animals, but it gradually became evident that this class of compounds may be more useful against skin, oral, and lung tumors.

*Canthaxanthin and lycopene as chemopreventive agents*

Canthaxanthin, a carotenoid with no vitamin A activity, was evaluated for its efficacy in the prevention of DMBA-induced mammary cancers in rats by Grubbs et al [44]. Diet supplementation with canthaxanthin for 3 weeks prior to the carcinogen resulted in a 65% reduction in the number of mammary cancers. Since the feeding of canthaxanthin after the administration of MNU had no significant effect on mammary carcinogenesis, it was concluded that this carotenoid is active in preventing cancer initiation and not promotion. These data suggest that vitamin A activity may not be required for carotenoids to be of value against mammary carcinogenesis.

Lycopene, a carotenoid from tomato, has been vigorously tested as a potential chemopreventive agent not only against mammary but also against prostate cancer. Recently, Nagasawa et al [45] examined the effect of lycopene on mammary tumor development using SHN virgin mice, a high mammary tumor strain. Lycopene when provided as a dietary supplement at the concentration of $5.0 \times 10^{-3}$M significantly suppressed mammary tumor development. Although a small decrease in body weight was evident in the lycopene treated group, no deleterious side-effects of lycopene were detected.

In human studies, improved survival was reported by Ingram [46] in a follow-up study of 103 breast cancer patients who consumed foods rich in beta-carotene (and vitamin C). In this study, only one death occurred in the group with the highest consumption of beta-carotene, compared to twelve deaths in the group that consumed the lowest levels of beta-carotene and eight deaths in the intermediate group. Since nutrient intakes were calculated based on the types and portions of foods these patients consumed after conventional therapy it is difficult to conclude that specific nutrients reduced mortality. The benefit is rather associated with increased fruit consumption, and it may be due to the presence of micronutrients that have not been measured. To our knowledge, there are no conclusive studies in which beta-carotene, in its purified form, prevented mammary carcinogenesis in animals or breast cancer in women.

**Conclusions**

Several retinoids have been found to be very effective against rat mammary carcinogenesis and one (4-HPR) is presently being evaluated in the clinic [40]. Among the carotenoids, on the other hand, beta-carotene has shown limited success in rodent models of mammary carcinogenesis and is of questionable value in the prevention of clinical breast cancer. Studies utilizing beta-carotene as a chemopreventive agent against breast cancer at this point are not merited, because only sporadic animal studies have been done and these have been particularly unsuccessful in demonstrating efficacy. However, other carotenoids such as canthaxanthin and lycopenes may be proved more effective against mammary carcinogenesis.

It is clearly evident that 4-HPR, as well as other retinoids, serve as effective chemopreventive agents against breast cancer. Finally, 4-HPR in combination with tamoxifen is more effective in suppressing breast cancer than either agent alone. It is becoming clear that retinoids have the potential to reverse the progression of malignancy and prevent carcinogenesis.

Thus, from the available data, it would appear that the antiproliferative effect of retinoids may prove beneficial in benign breast disease, and that
a clinical trial using a combination of 4-HPR and tamoxifen is warranted, at least in patients at high risk for developing breast cancer.

Acknowledgements

Sources of support: NIH Grant Nos. CA-62184 and CA-56785.

References

17. Dickens MS, Custer RP, Sorof S: Retinoid prevents mammary gland transformation by carcinogenic hydrocarbon in whole-organ culture. Proc Natl Acad Sci USA 76:5891-5895, 1979
26. Moon RC, Grubbs CJ, Sporn MB, Goodman DG:


