Dietary Fenretinide, a Synthetic Retinoid, Decreases the Tumor Incidence and the Tumor Mass of ras+myc-induced Carcinomas in the Mouse Prostate Reconstitution Model System


Scott Department of Urology, Baylor College of Medicine, Houston, Texas [K. S., D. K., S. H. P., P. T. S., T. C. T.]; The Urology Research Laboratory, Veterans Affairs Medical Center, Houston, Texas [K. S., D. K., S. H. P., T. C. T.]; Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 [T. C. T.]; and Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892 [M. A., M. B. S.]

Abstract

Several epidemiological studies have implicated low dietary and serum levels of retinol with an increased risk for the development of human prostate cancer. In a recent report, dietary fenretinide [N-(4-hydroxyphenyl)] retinamide], a synthetic retinoid with low toxicity, decreased the incidence of experimentally induced prostate cancer. Fenretinide is currently being evaluated in phase I and phase II clinical trials as an agent for both the treatment and chemoprevention of human prostate cancer. Because of these findings, we investigated whether dietary fenretinide could alter the incidence or phenotype of oncogene-induced prostate cancer in the mouse prostate reconstitution model system. When compared to control-fed animals, dietary fenretinide reduced the tumor incidence by 49% and the tumor mass by 52% of ras+myc-induced cancers in the mouse prostate reconstitution model system, which was modified to prolong the latency period before cancer development. Retinoids have a wide ranging effect on cellular differentiation, growth factor synthesis, and immune function. While its mechanism of action in this system remains unclear, fenretinide is an effective agent for the chemoprevention and growth modulation of oncogene-induced prostate cancer in the mouse prostate reconstitution model system and may be effective for the chemoprevention of human prostate cancer.

Introduction

Prostate cancer is now the second leading cause of cancer mortality and the leading incident non-skin cancer in U.S. males (1). Despite aggressive attempts at identifying and treating this disease at an early stage, prostate cancers have often progressed to a noncurable stage by the time surgical or radiation treatment is instituted. Prostate cancer represents an excellent candidate disease for chemoprevention because of its high prevalence and its presumed long latency period between initiation and the achievement of a fully malignant biological potential. Among the agents currently under use or consideration for prostate cancer chemoprevention trials are the 5-α reductase inhibitor, finasteride, and fenretinide, a synthetic retinoid (2). Despite the relative paucity of data concerning the effectiveness of these agents in animal models, the magnitude of the clinical problem in this country has generated great interest in proceeding with clinical trials.

Retinoic acid, synthesized from retinol, and its analogues comprise a family of compounds (retinoids) which act via a highly specific group of cytosolic binding proteins and nuclear receptors to regulate a wide range of responses including inhibition of cellular proliferation, cellular differentiation, morphogenesis, growth factor synthesis, immune stimulation, and extracellular matrix formation (reviewed in Ref. 3). Retinoids have demonstrated effectiveness in inhibiting tumor formation and metastasis in vitro and in vivo (4). Because of these characteristics, various retinoids have been evaluated in chemoprevention trials of human bladder cancer, lung cancer, and squamous cancers of the head and neck (5). The natural retinoids, all-trans-retinol (Vitamin A), all-trans-retinoic acid, 13-cis-retinoic acid, and retinyl palmitate, while exhibiting effectiveness in many of these trials, have limited potential as general chemopreventive agents because of their potential toxicity.

Fenretinide, or 4-HPR, is a retinoid which has demonstrated its clinical safety in a large ongoing breast chemoprevention trial in Europe (6, 7). Fenretinide has been shown to inhibit the formation of chemically induced breast cancer (2). In one study, fenretinide was able to inhibit the production of primary prostate cancers in a rat prostate cancer model (8). Furthermore, fenretinide was able to inhibit the growth of several rat and human prostate cancer cell lines in vitro and in vivo and to inhibit angiogenesis in a variety of assays (9). Few additional data are available on the effectiveness of fenretinide as a chemopreventive agent for prostate cancer. Because of this, we investigated whether dietary fenretinide could alter the incidence, phenotype, or behavior of oncogene-induced prostate cancer in the MPR model system.

Materials and Methods

Diet Preparation. One mmol of 4-HPR or fenretinide (McNeil Pharmaceuticals, Spring House, PA) was dissolved in 12.5 ml of ethanol, to which 37.5 ml Neobee oil (Stepan Co., Maywood, NJ), 1 ml of antioxidant, Tenox 5 (Eastman Chemicals, Kingsport, TN), and 3 μmol of Vitamin K (Sigma Chemicals, St. Louis, MO) were added. The retinoid solution was blended with 950 g of powdered commercial Purina 5002 m laboratory chow (Ralston Purina, St. Louis, MO) for 20–30 min and stored at 4°C in the dark. Control diet was prepared as described except 4-HPR was omitted. All animals were fed ad libitum.

Mouse Prostate Reconstitution. MPR tissues were generated as reported previously (10, 11). Briefly, urogenital sinus tissue was isolated from 17-day-old C57BL/6 mouse fetuses, and the anterior and posterior regions were trimmed leaving only that portion which gives rise to the prostate gland. After a 2-h digestion in 1.0% trypsin, standard microdissection techniques were used to separate the UGE from the UGM compartment. Previously we have shown that this technique yields less than 1% cross-contamination of cells between compartments. After the separation, both UGE and UGM were treated with 0.1% collagenase and washed, and an aliquot of the UGM suspension was counted. UGE cell numbers were extrapolated since the UGM:UGE cell ratio

Received 7/12/93; accepted 8/18/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was primarily supported by Grants CA50588 and SPORE P50-CA8204 from the National Cancer Institute and in part by the Veterans Administration. Additional support was provided by the American Foundation for Urologic Disease Research Scholar Program and the New York Academy of Medicine Ferdinand C. Valentine Fellowship Program.

2 To whom requests for reprints should be addressed, at 6535 Fannin, F427A, Houston, TX 77030.

3 The abbreviations used are: 4-HPR, N-(4-hydroxyphenyl)-retinamide; MPR, mouse prostate reconstitution; TOF, transforming growth factor; UGE, urogenital sinus epithelium; UGM, urogenital sinus mesenchyme.
has been shown previously to be 7:3. Transduction of the ras and myc oncogenes was accomplished via the replication deficient recombinant retrovirus Zipras/myc 9 (10). Zipras/myc 9 was derived from a Moloney murine leukemia virus-derived recombinant retroviral vector and contains the v-Ha-ras gene, as well as a fragment from MC29 carrying the v-gag/myc oncogene, both under the transcriptional regulation of the viral LTR promoter. Supernatants containing helper virus-free Zipras/myc 9 (5 X 10^5 focus forming units/ml) mixed with polybrene were added to UGE alone in these experiments, as opposed to both UGE + UGM in the classic MPR model (10), at a multiplicity of infection of approximately 1.0. Two h after infection, the UGE and UGM cells were pelleted. Then, 1.5 X 10^6 cells were mixed at the appropriate cell ratios to reconstitute the fetal urogenital sinus tissue and resuspended in collagen for an overnight incubation. The next morning, the collagen suspensions (MPRs) were grafted under the right renal capsules of adult isogenic male hosts. In some instances, hosts were double-grafted, with an MPR placed under the left renal capsule as well. Host mice were then segregated into equal groups, which were then fed either the fenretinide-containing or the control diet. Our previous studies indicated that animals fed the fenretinide-containing diet experienced no discernible adverse effects. After a 7-week in vivo growth period, MPRs were harvested, at which time the gross appearance and wet weight of the reconstituted prostate (MPR) was noted, and portions were snap-frozen in liquid nitrogen for RNA and DNA analysis, fixed in formalin for paraffin embedding, frozen in Tissue-Tek OCT medium (Miles Scientific) for cryostat sectioning, or placed directly into cell culture. Fifteen 3-μm sections were cut from the paraffin-embedded blocks from each MPR and the first and last 3 sections of each specimen were stained with hematoxylin and eosin, examined microscopically, and scored as either benign or malignant. As in previous studies, the possible spread of helper virus was monitored by performing reverse transcriptase assays on serum samples isolated from every individual host animal at the time of MPR tissue harvest (10, 11).

**Statistical Analysis.** Rates of tumor incidence were compared using χ^2 analysis and tumor weight was compared using an unpaired t test. All analyses were performed using the Statview 4.0 statistical package (Abacus Concepts, Inc.).

**Results**

The MPR model system uses recombinant retroviral vectors carrying oncogenes to initiate prostate cells, which are then reconstituted and grafted into male hosts for a period of in vivo growth. Depending on the oncogene(s) introduced, the mouse strain utilized, the compartment infected (epithelium versus mesenchyme, or both), and the length of the in vivo growth period before harvesting, a range of prostate phenotypes are reliably produced, including normal prostate, benign hyperplasia, dysplasia, prostatic intraepithelial neoplasia-like lesions, and frank prostate carcinoma as defined by both histological and immunohistochemical criteria (10–12). For example, when the ras+myc oncogenes are introduced into both the epithelial and mesenchymal compartments of C57BL/6 urogenital sinus tissue, carcinomas are produced in approximately 90% of cases within 4 weeks.

Because introduction of both the ras+myc oncogenes into both the epithelial and mesenchymal compartments leads to the rapid evolution of poorly differentiated prostate carcinomas in our system, and since human prostate cancer has a prolonged latency period, we modified the system to prolong the period between initiation and the development of cancer. By introducing the ras+myc oncogenes into UGE only, hyperplasias and dysplasias are evident after the usual 4-week in vivo growth period, but carcinomas are not produced at high frequency until the sixth or seventh week. We produced ras+myc initiated UGE-restricted MPRs and fed male host mice either a diet containing fenretinide or a control diet for a period of 7 weeks. In the control fed group (n = 13), carcinomas were produced in 11 MPRs, while 2 yielded hyperplasia only (tumor incidence = 85%). Seven mice in this group either died or were sacrificed before the end of the 7-week period because they appeared ill presumably due to tumor burden. In the fenretinide fed mice (n = 14), carcinomas formed in 6 MPRs and hyperplasias in 8 (tumor incidence = 43%), representing a 49% reduction in tumor incidence (P < 0.05). Only two mice in this group died or were sacrificed early. The mean tumor wet weight was 1.48 g in the control group and 710 mg in the fenretinide-fed group. These differences were also statistically significant (P < 0.05) (Table 1). While marked differences in tumor phenotype were not apparent between these two groups, a much higher incidence of morphological changes that resembled a premalignant phenotype was observed in the fenretinide-fed group (Fig. 1). Distant metastatic deposits were not detected in any of the host mice from the two groups, although full autopsies were always performed.

**Discussion**

Early experiments using mouse prostate explant cultures demonstrated that retinoic acid could both inhibit and reverse the proliferative effects of chemical carcinogens on prostatic epithelium (13, 14). Further interest in the ability of retinoids to inhibit prostate carcino genesis led to a number of retrospective case control studies attempting to correlate Vitamin A intake and serum levels with the risk of developing human prostate cancer (see examples in Refs. 15 and 16). These studies have yielded conflicting results, with one study even suggesting that Vitamin A was associated with an increased risk of developing prostate cancer in men over 70 years of age (17). Due to the relative lack of appropriate animal models for studying the processes underlying the development and progression of prostate cancer, the effectiveness of retinoids in general, and fenretinide in particular, in prostate cancer chemoprevention has not been adequately evaluated. We have now demonstrated that fenretinide reduces both the tumor incidence and tumor mass of ras+myc-induced cancers in the MPR model system, modified to increase the latency period before cancer development by leaving the stromal compartment intact.

The MPR model system, which uses transduction of the ras and myc oncogenes to initiate prostate cells, is uniquely suited for studying the molecular events underlying prostate carcinogenesis and has yielded insights which have been directly applicable to human prostate cancer. Activated ras alleles are a common feature in many primary human cancers and have been detected in up to 25% of primary human prostatic cancers (18–21). Elevated levels of c-myc expression have also been associated with primary human prostate cancer.
cancer (22, 23). In addition, these two oncogenes interact with a host of other gene products which regulate cellular growth and differentiation and may play an as yet undetermined role in prostate carcinogenesis. In the MPR model system, susceptibility to oncogene-induced prostate carcinogenesis is dependent on the mouse strain utilized. For example, while the C57BL/6 strain is highly susceptible to the development of prostate cancer in this model, BALB/c mice are resistant, developing cancer only at low frequency. The importance of genetic background on cancer susceptibility in this model reflects the epidemiology of human prostate cancer, in which the clinical incidence varies almost 20-fold between Japanese and American men, and by 1.5–2-fold between black and white Americans (24).

We have shown previously that in the MPR model system, the differences in strain susceptibility to oncogene-induced prostate carcinogenesis may be determined by genetic differences in mesenchyme (12). We have also shown that in this model system, mesenchymal accumulation of TGF-β1 occurs before the development of prostate carcinoma, and accumulation of this growth factor occurs in and around tumor cells thereafter (11).4 These findings have been extended to human prostate cancer, in which marked focal stromal accumulation of TGF-β1 has been demonstrated in BPH nodules4 and within prostate cancers (25). Another recent study demonstrated that dermal fibroblasts produce a diffusible inhibitory factor belonging to the TGF-β family, possibly TGF-β3, that can suppress the growth of ras-transformed primary keratinocytes (26). Overall these data suggest that specific members of the TGF-β family may mediate carcinogenesis in a paracrine fashion.

Retinoids have been shown in a variety of systems studied to stimulate synthesis of the β family of transforming growth factors, especially TGF-β2, suggesting that modulation of TGF-β represents one possible mechanism for the observed inhibition of prostate carcinogenesis (27). Studies that address this possibility are currently underway. In addition to the possible intermediary role of TGF-β, some of the effects of retinoids on prostate carcinogenesis may be mediated through other intrinsic or host-mediated pathways. Retinoic acid has been shown to inhibit the activity of 5α-reductase, the enzyme that converts testosterone to its highly active metabolite, dihydrotestosterone in the prostate (28, 29). Finally, retinoids have been found to play an important role in regulation of the immune system, including enhancement of interleukin 2-induced LAK cell formation (30).

While fenretinide treatment led to a dramatic reduction in both the tumor incidence and tumor mass of prostate cancer in our model system, a significant proportion of the prostate reconstitutions in this system developed carcinoma despite treatment. This may be explained by a number of possibilities. While the drug dosage and route of administration in these experiments were well tolerated by the treated animals, the optimal dosage and administration is still unclear and a more pronounced effect may be possible utilizing an alternative schedule. In addition, while modification of the MPR model system (epithelial infection only versus epithelial plus mesenchymal infection) increases the latency of cancer formation from 4 to 7 weeks, the introduction of 2 potent oncogenes into prostatic epithelial cells remains a powerful stimulus towards carcinogenesis and may lead to early progression towards malignancy despite the negative effects of fenretinide on this process. Finally, it is conceivable that both human prostate cancer and its counterpart, oncogene-induced mouse prostate cancer, may develop along multiple molecular pathways. In the MPR model system, some of these pathways may be influenced by fenretinide but others may not.

Human prostate cancer presents an excellent opportunity for chemoprevention. As a consequence of the long latency period required for cancer progression and the large population of men thought to harbor latent forms of prostate cancer, effective chemopreventive agents have a high likelihood of impacting favorably on this disease. Because older men are primarily affected, small effects on the rate of progression rather than complete inhibition of cancer formation may be all that is required for a successful chemopreventive strategy. While the molecular events underlying this progression remain to be determined, we are beginning to accumulate enough knowledge about the overall biology of both normal and neoplastic prostate growth to begin attempts at modulating this process. We have presented additional evidence that fenretinide may be an effective agent in the chemoprevention of prostate cancer, decreasing both tumor incidence and tumor mass in our experimental system. Further studies of fenretinide utilizing this system may help elucidate its mechanism of action and the effect of temporal factors, increasing the body of knowledge which will be required to design effective human trials.

References


4 Unpublished results.
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