Transforming growth factors-β are not good biomarkers of chemopreventive efficacy in a preclinical breast cancer model system

ABSTRACT

Using a carcinogen-initiated rat model of mammary tumorigenesis, we tested the hypothesis that transforming growth factor (TGF)-βs are useful biomarkers of chemopreventive efficacy in the breast. The chemopreventive agents tested were tamoxifen and the retinoids 9-cis-retinoic acid (9cRA) and N-(4-hydroxyphenyl)retinamide (4-HPR), because both antiestrogens and retinoids have previously been shown to upregulate TGF-βs in vitro. Despite demonstrable chemopreventive efficacy in this model, none of these agents, alone or in combination, had any significant impact on the expression of TGF-βs in the mammary ductal epithelium or periductal stroma as determined by immunohistochemistry. These data suggest that TGF-βs are not likely to be useful biomarkers of chemopreventive efficacy in a clinical setting.

INTRODUCTION

Synopsis Introduction: Chemoprevention has been defined as the use of noncytotoxic nutrients or pharmacologic agents to enhance intrinsic physiologic mechanisms that protect the organism against the development of mutant clones and their progression to malignant cancer. In a recent landmark trial, tamoxifen, a hormonally active selective estrogen receptor modulator (SERM), was shown to decrease the risk of invasive breast cancer by 49% in asymptomatic, but at-risk women. The search is now on for agents with improved risk-benefit profiles, and for agents that will prevent the subclass of estrogen receptor-negative tumors, the incidence of which was unaffected by the SERMS. Retinoids have already shown potential in this regard. Because it will not be possible to test many agents in large randomized clinical trials, efforts are underway to develop useful tissue-based surrogate end-point biomarkers that can be used to select only the most promising agents (and doses) for large-scale trials. Provocative mechanistic connections have been made between the steroid hormone superfamily, including the SERMS and retinoids, and the TGF-β family of multifunctional growth factors. The TGF-β system has tumor suppressor activity, and loss of TGF-β response is associated with advanced disease in many human tumor types, including the breast. Conversely, experimental overexpression of TGF-β in the mammary gland protects against tumorigenesis. This strongly suggests that interventions that enhance TGF-β function early in tumorigenesis could delay or prevent the course of the disease. SERMs such as tamoxifen can upregulate TGF-β production and activation by many cell types, including human breast cancer cell lines. Similarly, retinoids can upregulate TGF-β production and activation, both in cell culture and in rats in vivo. It is plausible, therefore, that upregulation of endogenous TGF-β could contribute to the chemopreventive efficacy of SERMs and retinoids. In the present study we used a carcinogen-induced rat mammary carcinogenesis model to test the hypothesis that chemoprevention by tamoxifen and retinoids is associated with local upregulation of TGF-βs in the mammary gland, and that TGF-βs might therefore be useful as potential surrogate end-point biomarkers of chemopreventive efficacy in clinical trials. Materials and methods: A standard protocol for induction of breast cancer in female Sprague-Dawley rats using a single dose of N-nitroso-N-methylurea (NMU) at 8 weeks of age was used. Chemopreventive agents were incorporated into powdered lab chow and fed ad libitum, beginning 1 week after injection with NMU. The rats were

fed 9cRA (Kuraray Company, Osaka, Japan) at 120 mg/kg of diet, tamoxifen (Sigma Chemical Co, St Louis, MO, USA) at 1.0 mg/kg of diet, and 4-HPR (RW Johnson Pharmaceutical Research Unit, Spring House, PA, USA) at 782 mg/kg of diet. Rats were weighed and palpated for the presence of mammary tumors weekly, and six rats in each experimental group were sacrificed after 6 and 12 weeks of treatment with chemopreventive agent. For experiments to determine the effect of high doses of tamoxifen administered over shorter periods of time, rats were given 10 mg tamoxifen/kg body weight per day intragastrically, or 1 mg tamoxifen/kg in the diet, and were sacrificed after 1 day or 3 weeks of treatment. All palpated tumors were confirmed at necropsy, and mammary glands were fixed in neutral buffered formalin and embedded in paraffin. The number 2 (first thoracic) mammary gland was sectioned for histology and immunohistochemistry. Immunohistochemical staining was done using rabbit polyclonal antibodies raised against synthetic peptides that correspond to regions in the mature forms of TGF-β1, TGF-β2 and TGF-β3: anti-TGF-β1-LC and anti-TGF-β1-CC, anti-TGF-β2 (sc-90; Santa Cruz Biotechnologies Inc, Santa Cruz, CA, USA), and anti-50-60-β3-LC, respectively. Anti-latent TGF-β-binding protein (LTBP; Ab39) was raised against the purified full-length platelet LTBP. The antibodies were affinity purified against the immunizing peptide (anti-TGF-β3) or against protein A sepharose (anti-TGF-β1-LC, anti-TGF-β1-CC and anti-TGF-β2). Immunohistochemical staining was performed using an indirect immunoperoxidase detection protocol (Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA). Staining intensity was scored on a scale of 0-4+, using the mouse embryo control section as a reference standard for each run. Ducts and periductal stroma were scored independently. Staining was scored in a blinded manner by two independent observers, and discrepancies were rescored by consensus. Staining intensity was plotted as the mean ± standard deviation for each experimental group. Results: Palpable mammary tumors were first detected after approximately 35 days following initiation of NMU, and by 70 days incidence had reached 100% in rats not treated with chemopreventive agents (Fig. 1a). Tamoxifen, alone or in combination with retinoids, decreased tumor incidence by more than 70% by the end of the study, whereas 9cRA alone decreased it by 50%. 4-HPR alone had a relatively modest effect on tumor incidence in the present study. However, it significantly decreased tumor multiplicity (Fig. 1b), indicating that the dose used was efficacious. There was minimal toxicity associated with the chemopreventive intervention, except in the tamoxifen + 4-HPR group, in which mild toxicity was observed, as judged by the weights of the experimental animals (Fig. 1c). All three TGF-β isoforms and the LTBP (part of the naturally occurring latent TGF-β complex) showed broadly similar immunostaining patterns in the mammary glands of untreated rats at 15 weeks of age (Fig. 2). They were present both in the ductal epithelium and in the periductal stroma, suggesting that the TGF-βs are synthesized by the epithelial cells, and possibly stromal cells, and are sequestered in the extracellular matrix. This staining pattern is consistent with a role for the TGF-βs in the maintenance of normal mammary homeostasis. None of the chemopreventive agents used, alone or in combination, were found to affect expression of any of the TGF-β isoforms or the LTBP in either ductal epithelium or periductal stroma after 6 weeks of chemopreventive intervention (Fig. 3). The 6-week time point was chosen as representative of the period of preneoplasia, as the majority of the animals had no palpable tumors at this time (Fig. 1). In the study set, eight out of 36 (22%) of the slides showed histologic evidence of hyperplasia, one out of 36 had a ductal carcinoma in situ (mammary intraepithelial neoplasia), and one out of 36 had a carcinoma. We further

investigated the effect of tamoxifen at higher doses and earlier time points. In rats that received tamoxifen at 10 mg/kg per day intragastrically (equivalent to 600 mg/day for a human) or 1 mg/kg per day intragastrically (equivalent to 60 mg/day for a human) for either 1 day or 3 weeks, again no consistent changes were seen in TGF-β expression, using either the TGF-β1-CC or the TGF-β2 antibodies (data not shown). After 6 weeks of treatment, we noticed that mammary glands from tamoxifen-treated rats were less developed than those of untreated control animals, having fewer tertiary ducts and terminal end buds, and they could consistently be identified from a blind data set (Fig. 4). By 12 weeks of treatment, all three chemopreventive agents had a significant effect on glandular histology, with tamoxifen and 9cRA showing the greatest suppression of ductal development and lobule formation, and 4-HPR showing a relatively mild effect. Discussion: One major goal in the field of prevention is the identification of surrogate biomarkers that might rapidly predict the effect of a given agent on the primary end-point of cancer incidence. The most informative markers are those with modulation that is likely to be directly related to the preventive effect, and a compelling argument can be made that TGF-βs may fall into this category. However, the present data in a well-established preclinical model of breast cancer, employing a variety of highly effective chemopreventive regimens, suggest that this is not the case. Most of the previous studies on the regulation of TGF-βs by tamoxifen and retinoids have been done in tissue culture. The lack of effect on TGF-β expression in the present in vivo study may reflect the dependence of the response on contextual cues that are only present in the artificial in vitro environment. In an in vivo study, all-trans-retinoic acid was shown to cause an upregulation of TGF-β isoforms in rats, with kinetics and isoform selectivity that varied with the target tissue. However, the rats were vitamin A-deficient, and it is not known whether the same effects would be seen in vitamin A-replete animals such as were used in the present study. In a small study in humans tamoxifen treatment was shown to cause a consistent induction in extracellular TGF-β in breast cancer biopsies, when compared with pretreatment biopsies from the same patients, and complex effects of tamoxifen on induction of TGF-β2 in the plasma of patients with metastatic breast cancer have been described. It is possible that tamoxifen is only effective in inducing TGF-β in the context of a tumor, and not in the normal or initiated tissue that was the subject of the present study. However, an optimal surrogate end-point biomarker in a prevention setting needs to be modulated in normal or premalignant tissues. Although we cannot eliminate the possibility of more subtle effects of chemopreventive agents on TGF-β bioavailability or cellular responsiveness, in our preliminary analyses we have seen no effects on the expression of type I and type II TGF-β receptors (data not shown). There is considerable evidence to suggest that, at late stages in tumorigenesis, TGF-βs can actually promote the tumorigenic process, particularly if the epithelial cells have lost responsiveness to the growth inhibitory effects of TGF-β by this time. While the present work was in progress, a study was reported that showed that loss of the type II TGF-β receptor can already be seen in a significant fraction of hyperplasias without atypia in the human breast. Furthermore, loss of the receptor correlated with increased risk of subsequent development of invasive breast cancer. Thus, loss of TGF-β response may be a very early event in the development of human breast cancer. Because locally elevated TGF-β levels could select for TGF-β-resistant cells, and because TGF-βs can have oncogenic effects on the stroma, it may actually be important for the safety profile of chemopreventive agents to demonstrate that they do not increase TGF-β levels in the at-risk breast. In this regard, this demonstration that the expression of TGF-βs in the preclinical rat model is

unaffected by tamoxifen, 9cRA, or 4-HPR may actually have positive implications, because all three agents are already in clinical use. The NMU-induced rat model of mammary tumorigenesis is widely used for chemoprevention studies, and yields rapid development of hormonally responsive mammary tumors with 100% incidence. To do this, the initiating agent is given at 8 weeks of age and the chemopreventive agent is started a week later, during the period of active development of the mammary gland. We observed that the histology of the tamoxifen-treated mammary glands differed significantly from control glands when examined after 6 weeks of tamoxifen treatment, showing fewer terminal end-buds and less tertiary branching. Part of the chemopreventive efficacy of antiestrogens and retinoids in this model may therefore be due to a generalized decrease in ductal development. Since chemopreventive agents are unlikely to be given to humans during the pubertal period, this form of preclinical model may not accurately reflect the degree of chemopreventive benefit that could be achieved in humans. Although the accelerated time course and high penetrance of disease reduces the costs of this model, it may be advisable to confirm efficacy of promising agents in a model that delays application of the chemopreventive agent until the mammary gland is fully developed. In conclusion, we have shown that treatment of rats with tamoxifen or retinoids results in effective chemoprevention of mammary tumorigenesis, without any detectable effect on local expression of TGF-βs. Although we cannot rule out more subtle effects on TGF-β activity, such as the activation of latent forms, the data suggest that TGF-βs are not involved in the underlying molecular mechanism of chemoprevention induced by these agents. This agrees with in vitro work that showed that blockade of TGF-β signaling did not abrogate the growth inhibitory effect of tamoxifen on breast cancer cells. Given the very limited breast tissue available in clinical trials, we do not recommend testing for TGF-βs as a surrogate end-point biomarkers at this time. Full article Introduction Chemoprevention has been defined as the use of noncytotoxic nutrients or pharmacologic agents to enhance intrinsic physiologic mechanisms that protect the organism against the development of mutant clones and their progression to malignant cancer. Members of the nuclear receptor superfamily are considered to be particularly promising targets for chemoprevention, because of their pivotal role in the regulation of metabolic, developmental, and differentiation pathways. In a recent landmark trial, tamoxifen, a hormonally active SERM, was shown to decrease the risk of invasive breast cancer by 49% in asymptomatic, but at-risk women. Another SERM, raloxifene, also shows promise. These studies validate the concept of using pharmacologic agents for prevention of human breast cancer in apparently healthy individuals. The search is now on for agents with improved risk-benefit profiles, and for agents that will prevent the subclass of estrogen receptor-negative tumors, the incidence of which was unaffected by the SERMS. Retinoids, a family of compounds structurally related to vitamin A, have already shown potential in this regard. Since it will not be possible to test many agents in large randomized clinical trials, efforts are underway to develop useful tissue-based surrogate end-point biomarkers that can be used to select only the most promising agents (and doses) for large-scale trials. Provocative mechanistic connections have been made between the steroid hormone superfamily, including the SERMS and retinoids, and the TGF-β family of multifunctional growth factors. TGF-βs are potent inhibitors of the growth of many epithelial cell types. Recent work has implicated the TGF-β system as an important tumor suppressor pathway, and loss of TGF-β response is associated with advanced disease in many human tumor types, including the breast. In mouse models, over-expression of TGF-β1 in the mammary gland protects against tumorigenesis, whereas local

inactivation of the type II TGF-β receptor enhances tumorigenesis. This strongly suggests that interventions that enhance TGF-β function early in tumorigenesis could delay or prevent the course of the disease. Antiestrogens such as tamoxifen have been shown to upregulate TGF-β production and activation by many cell types, including human breast cancer cell lines. Similarly, retinoids can upregulate TGF-β production and activation, both in cell culture and in rats in vivo. Therefore, it is reasonable to propose that some of the chemopreventive efficacy of these agents against breast cancer in vivo could be mediated via a local upregulation of TGF-βs, with concomitant enhancement of tumor suppressor activity. In the present study, we used a carcinogen-induced rat model of mammary carcinogenesis to test whether chemoprevention by tamoxifen and by two different retinoids (4-HPR, also known as fenretinide; and 9-cRA) is associated with local upregulation of TGF-βs in the initiated mammary gland. If this were the case, TGF-βs might be useful as potential surrogate end-point biomarkers in clinical trials. However, the results show that TGF-β levels, as detected immunohistochemically, are not affected by tamoxifen or retinoids in this preclinical model of early-stage breast cancer.

CONCLUSION

We have shown that treatment of rats with tamoxifen or retinoids results in effective chemoprevention of mammary tumorigenesis, without any detectable effect on local expression of TGF-βs. Although we cannot rule out more subtle effects on TGF-β activity, such as the activation of latent forms, the data suggest that the underlying molecular mechanism of chemoprevention by these agents does not involve increases in TGF-β expression. This agrees with in vitro work showing that blockade of TGF-β signalling did not abrogate the growth inhibitory effect of tamoxifen on breast cancer cells. Given the very limited breast tissue available in clinical chemoprevention trials, we do not recommend testing for TGF-βs as surrogate end-point biomarkers at this time.