Progressive Decrease in Nuclear Retinoic Acid Receptor \( \beta \) Messenger RNA Level during Breast Carcinogenesis\(^1 \)

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Abstract

Some of the nuclear retinoic acid receptors (RARs) \( \alpha, \beta, \gamma \) and retinoid X receptors (RXRs) \( \alpha, \beta, \gamma \) are thought to mediate the effects of retinoids on cell growth, differentiation, and apoptosis and thereby prevent breast carcinogenesis. We analyzed the expression of mRNAs for the three RARs and RXR-\( \alpha \) in histological sections of specimens from 70 breast cancer patients, which included adjacent normal tissue, ductal carcinoma in situ, and invasive cancer, using in situ hybridization. RARs \( \alpha, \beta, \gamma \) and RXR-\( \alpha \) were expressed in 98.1, 98.0, 93.6, and 100% of the adjacent normal tissues. Significant decreases in the number of cases expressing RAR-\( \beta \) were observed among ductal carcinoma in situ (83.1%) and invasive carcinomas (51.6%), especially among the poorly differentiated cases (77.4 and 35.7%, respectively). No relationship was found between the expression of estrogen receptor and RAR-\( \beta \). These results implicate decreases in RAR-\( \beta \) expression in breast cancer development and suggest that they are independent of estrogen receptor status.

Introduction

Breast cancer is the most common malignant neoplasm in women, accounting for 32% of all female cancers. In 1997, it is estimated that 181,600 new cases of breast cancer will be diagnosed in the United States (1). Despite improvements in early diagnosis of and surgical and adjuvant systemic therapies for breast cancer, the mortality rate has remained high and will result in 44,190 deaths in 1997. It is therefore necessary to continue searching for novel approaches to breast cancer prevention and treatment.

Chemoprevention could be used to reduce breast cancer morbidity and the occurrence of second primary tumors. Retinoids, structural and functional analogues of vitamin A, occupy a prominent position among the chemopreventive agents that have been examined for activity against breast cancer development in preclinical studies (2-4) and a clinical trial (5), although they were found to be ineffective against advanced breast cancer (6).

It is thought that the ability of retinoids to modulate in vitro malignant breast cancer cell proliferation (7), apoptosis (8), and differentiation (9) may be related to their ability to suppress mammary carcinogenesis in vivo. The effects of retinoids are mainly mediated by two classes of nuclear retinoid receptors that are members of the steroid hormone receptor superfamily that also includes estrogen, thyroid hormone, and vitamin D receptors (10, 11). The nuclear retinoid receptors are divided into RARs\(^4 \) and RXRs that are both composed of three subtypes (\( \alpha, \beta, \gamma \)). RARs bind all-trans-retinoic acid and 9-cis-retinoic acid, whereas RXRs bind only the latter retinoid. RARs can form heterodimers with RXRs, bind to specific DNA sequences, RA response elements, in the promoter regions of genes and, upon activation by ligand, enhance the transcription of target genes. Each of the subtypes exhibits specific patterns of expression during embryonal development and different distributions in adult tissues; therefore, they are thought to regulate distinct genes (10, 11). This contention is supported by recent findings that the regulation of specific genes is abrogated in cells in which specific receptors have been knocked out (12).

Defects in retinoid receptor structure, expression, and function have been detected in various types of cancer cells in vitro (13) and in vivo (14-16), and it has been suggested that they may enhance cancer development by interfering with retinoid signaling, thereby abrogating the putative physiological anticarcinogenic effects of natural retinoids.

In this study, we describe for the first time a comparison of the expression of RARs and RXR-\( \alpha \) in adjacent normal breast epithelial tissue, the premalignant breast tissue DCIS, and invasive breast cancer. We show that RAR-\( \beta \) expression is lost in about 50% of the invasive cancers compared to normal and DCIS tissues and provide support for the idea that altered expression of RAR-\( \beta \) may be involved in the pathogenesis of breast cancer.

Materials and Methods

Surgical Specimens. Tissue samples were obtained from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center. These samples were routinely fixed in 10% buffered formalin and embedded in paraffin. The specimens were cut into 4-\( \mu \)m sections and stained with H&E for classification as normal, low-grade (well differentiated; grades 1 and 2 combined), or high-grade (poorly differentiated; grade 3) DCIS and low- or high-grade invasive cancer. Samples were selected by the pathologist (N. S.) based on histological diagnosis to ensure that a tumor and adjacent normal epithelium or DCIS were present.

In Situ Hybridization. A nonradioactive in situ hybridization using digoxigenin-conjugated antisense riboprobes was used exactly as described by us previously (14, 15). The quality and specificity of the probes were determined by Northern blotting using extracts of cultured cells with known receptor expression pattern, and the specificity of the binding of antisense riboprobes was verified using sense riboprobes.

Immunohistochemistry. The immunohistochemical detection of ER was performed by a modification of the ABC technique as described previously by us (17), except that the glass slides containing sections selected for ER detection were placed in a microwave oven for 15 min in 0.01 M citric acid solution for antigen retrieval before staining with the ER antibody. Controls

\(^{4}\) The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; DCIS, ductal carcinoma in situ; ER, estrogen receptor; RA, retinoic acid.

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included incubation with normal mouse IgG instead of primary antibodies or with second antibody only. These controls were negative.

Data Analysis. The results of the in situ hybridization and immunohistochemical staining were reviewed by two pathologists who observed the sections using a Nikon microscope. Because the intensity of the in situ hybridization staining of the tissues was usually fairly uniform, and most cells in a given microscopic field exhibited a similar staining, a tissue was determined to be either positive or negative for retinoid receptor expression. A specimen was considered positive for ER when more than 10% of the epithelial cells were positive. For statistical analysis, the McNemar test was performed to determine the association between the expression of nuclear retinoid receptors and histology in matched pairs such as the one between tumors and adjacent normal tissues or DCIS. The \( \chi^2 \) test was applied to examine the association of two variables in unmatched samples.

Results

Detection of Nuclear Retinoid Receptor Expression in Normal, DCIS, and Malignant Breast Tissues by In Situ Hybridization. Surgical specimens from 70 breast cancer patients used in this study included mostly stage T1 and T2, and about 60% had lymph node involvement (Table 1). These specimens contained invasive carcinomas (n = 66), adjacent normal mammary tissues (n = 60), and DCIS (n = 63). The expression of nuclear retinoid receptors in these specimens was detected by in situ hybridization. Examples for the results of such an analysis performed on consecutive histological sections of a sample from one patient that included adjacent normal gland, comedo-type DCIS, and invasive cancer are shown in Fig. 1. It demonstrates that all the nuclear receptors analyzed were expressed in the adjacent normal tissue and in the DCIS. In contrast, the level of expression of RAR-\( \beta \) and, to a lesser extent, RAR-\( \alpha \), was much lower in the invasive cancer than in the adjacent DCIS or normal cells. ER staining in this sample was positive in the normal gland but negative in the DCIS and the invasive cancer.

The results of the analysis of numerous specimens are presented in Table 2. The adjacent normal tissues expressed all the nuclear retinoid receptors in nearly all cases. The expression of RAR-\( \alpha \), RAR-\( \gamma \), and RXR-\( \alpha \) in DCIS was similar to that of normal tissue; however, the expression of RAR-\( \beta \) was significantly lower in DCIS than in normal tissue, especially in the poorly differentiated (high-grade) lesions (Tables 2 and 3). The expression of RAR-\( \gamma \) and RXR-\( \alpha \) in invasive cancer was similar to that in DCIS and adjacent normal tissue, whereas the expression of RAR-\( \alpha \) and RAR-\( \beta \) decreased to 83.6 and 51.6%, respectively, in invasive carcinomas. The decreased expression of RAR-\( \alpha \) in invasive carcinomas compared to normal as well as DCIS was of borderline significance (\( P = 0.059 \) and 0.046 by McNemar test, respectively), whereas the decrease in RAR-\( \beta \) expression compared to normal as well as DCIS was significant (\( P < 0.001 \) by McNemar test). Among the cases with DCIS or invasive cancers, those that were classified as poorly differentiated (high grade) showed a lower percentage of cases expressing RAR-\( \beta \) than did the well-differentiated (low grade) lesions (Tables 2 and 3). Thus, our results show a clear gradual decrease in RAR-\( \beta \) expression from 98.0% positive cases among normal tissues to 77.4% in high-grade DCIS, to 35.7% in high-grade invasive cancer. Though not statistically significant, a trend toward lower expression of RAR-\( \gamma \) and RXR-\( \alpha \) was observed in the high-grade invasive cancers.

To assess the correlation between RAR-\( \beta \) loss and nodal involvement, 65 patients for whom data were available on both the nodal status and RAR-\( \beta \) expression were analyzed. The data indicated that 59% (17 of 29) of the patients with loss of RAR-\( \beta \) expression in tumor, DCIS, or adjacent normal tissue had nodal involvement compared to 67% (24 of 36) of patients with intact RAR-\( \beta \) having nodal metastasis. The \( \chi^2 \) test showed no statistically significant correlation (\( P = 0.50 \)) between RAR-\( \beta \) expression and nodal status.

Expression of ER and Its Correlation with the Expression of RARs. The expression of ER was analyzed using immunohistochemical staining (Fig. 1). ER was detected in 81.7% of the adjacent normal specimens, but the expression decreased in DCIS and invasive cancer (Table 2). Among DCIS and invasive cancer specimens, those that were classified as poorly differentiated (high grade) showed greater losses of ER expression than those that were classified as well differentiated (low grade), and this difference seemed to be statistically significant (Table 2). The expressions of RAR-\( \alpha \), RAR-\( \beta \), and RAR-\( \gamma \) among the 19 invasive cancer specimens that were ER- were 78.9, 47.4, and 88.2%, respectively, whereas the expressions of these receptors among the 43 ER+ cases were 85.7, 53.5, and 88.6%, respectively. Thus, the expression of retinoid receptors did not seem to be related to ER status.

Discussion

Epidemiological studies suggest that physiological retinoids may play a role in suppressing breast cancer development (18). Retinoids may affect breast carcinogenesis by regulating the proliferation and differentiation of normal breast epithelial cells (19) and malignant breast cancer cells (9), by inducing apoptosis (8), and by suppressing properties associated with tumor aggressiveness (20, 21). Because nuclear retinoid receptors are thought to mediate most of the effects of retinoids on gene expression, reduced expression of one or more of these receptors may abrogate retinoid signaling and result in enhanced cancer development.

Two previous studies examined RAR-\( \alpha \) expression in vivo in breast cancer specimens. One study analyzed RAR-\( \alpha \) and ER mRNA expression in 116 primary breast tumors by Northern blot analysis and reported that RAR-\( \alpha \) was expressed in 87 of 94 ER+ tumors (22). The coexpression of ER and RAR-\( \alpha \) suggested that RAR-\( \alpha \) expression may be modulated in breast cancer cells by estradiol (22). However, the other study, which assessed the presence of RAR-\( \alpha \) protein in 33 breast lesion specimens by immunohistochemical analysis with RAR-\( \alpha \) antibody, demonstrated that the nuclear staining for RAR-\( \alpha \) in tumor tissue and adjacent normal epithelial cells was independent of ER protein levels (23). Northern blotting using total RNA from 5 ER+ and 6 ER- breast carcinoma cell lines revealed that RAR-\( \alpha \) was expressed in all cell lines, but its levels were greater in the ER+ cell lines. RAR-\( \gamma \) mRNA was expressed in all cell lines at levels that were independent of ER status (24).

Our study used in situ hybridization for detecting the mRNAs for the three RARs and RXR-\( \alpha \) in surgical specimens containing adjacent normal breast epithelium, DCIS, and invasive cancer. We have dem-
Fig. 1. Detection of RARs, RXR-α, and ER in adjacent normal human breast tissue, DCIS, and invasive cancer. Consecutive histological sections from a single tissue block from one patient that included adjacent normal gland, comedo-type DCIS, and invasive carcinoma were analyzed by nonradioactive in situ hybridization using digoxigenin-conjugated antisense riboprobes to detect retinoid receptor mRNAs, and immunohistochemistry was used to detect ER protein.

Our report presents the first analysis of RAR-β expression in vivo. We found that RAR-β expression is lost in nearly 50% of invasive breast carcinomas. This finding is similar to our previous findings with head and neck squamous cell carcinomas (14) and non-small cell lung carcinomas (15). In head and neck lesions, the loss of RAR-β expression was observed in 44% of dysplasias and 65% of squamous cell carcinomas (14). In the breast tissues, the loss of RAR-β expression was 22.6% in poorly differentiated DCIS and 64.3% in poorly differentiated breast carcinoma. This suggests that the loss of RAR-β expression is a later event in breast carcinogenesis than it is in head...
and neck carcinogenesis. The in vivo data are in partial agreement with in vitro studies with established breast carcinoma cell lines, which have shown that RAR-β mRNA was present in 7 of the 11 lines tested (loss in 36% of cell lines; Ref. 24). Another study failed to detect RAR-β expression in any of five breast carcinoma cell lines by Northern blotting but found that RA treatment could induce RAR-β expression in the two estrogen-dependent but not in any of the three estrogen-independent cell lines (25).

Our findings support the hypothesis that the loss of RAR-β expression may be related to breast cancer development, for example, by enhancing proliferation and resistance to apoptosis. This hypothesis is based on previous findings, which have demonstrated that induction of RAR-β by RA correlated with the growth-inhibitory effect of retinoids and that RAR-β mediated RA induced growth inhibition and apoptosis after transfection into estrogen-independent retinoic acid-resistant breast carcinoma cell lines (25–28). Furthermore, hormone-dependent retinoid-responsive breast carcinoma cells that expressed transfected RAR-β antisense RNA showed diminished responsiveness to RA (28). Introduction of RAR-α also restored response to RA in hormone-independent cells, but this was due to the induction of endogenous RAR-β (28).

Although decreased expression of RAR-β seems to be a common event in breast carcinogenesis as well as in the development of other tumor types, the underlying molecular mechanism is unclear. The RAR-β gene promoter includes a RA response element, which can be activated by RXR-RAR heterodimers (10, 11). However, many breast cancer cell lines exhibit defects in activation of the RAR-β promoter, or to defective retinoid metabolism that could decrease retinoid intracellular levels such that induction of endogenous RAR-β is compromised.

In conclusion, we have demonstrated that the expression of RAR-β is diminished progressively during breast carcinogenesis, specifically in high-grade DCIS lesions and invasive breast cancers. We propose that the loss of RAR-β expression may play a role in the conversion of DCIS to invasive cancer and the acquisition of aggressive properties of invasive breast carcinoma.

References


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