Loss of Retinoic Acid Receptor β Expression in Breast Cancer and Morphologically Normal Adjacent Tissue but not in the Normal Breast Tissue Distant from the Cancer

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Abstract

Retinoids and their receptors [retinoic acid receptors (RARs) and retinoid X receptors] play an important role in maintaining the balance between proliferation and apoptosis. Recently, Deng et al. [Science (Washington DC), 274: 2057-2059, 1996] reported a loss of heterozygosity on chromosome 3p24 in breast cancer specimens and the morphologically normal appearing adjacent tissue. The 3p24 locus includes, among other genes, the region coding for RAR-β. This study was designed to determine whether there are abnormalities in the expression of retinoid receptors in surgical specimens of patients with breast cancer.

In 14 patients, transcripts of nuclear retinoid receptors were detected by in situ hybridization in formalin-fixed, paraffin-embedded specimens by means of digoxigenin-labeled riboprobes specific for RAR-α, -β, and -γ. We found RAR-α expressed in all specimens, whereas RAR-γ was expressed in 100% of normal breast tissue but in only 11 of 14 tumorous lesions. RAR-β was found in all cases of normal breast tissue localized distant from the tumor, but in 13 of 14 cases it was completely absent in the tumor and the morphologically normal appearing tissue adjacent to the tumor. One possibility to explain the suppression of RAR-β is a mutation in the promoter region. Sequencing the DNA extracted from paraffin-embedded tumor tissue of the corresponding breast cancer specimens, we were not able to detect any mutation in the retinoic acid-responsive element.

Our results clearly indicate a crucial role of RAR-β in the carcinogenesis of breast cancer.

Introduction

Breast cancer is diagnosed in 182,000 women each year, and ductal carcinoma in situ is diagnosed in an additional 25,000 in the United States. The incidence of breast cancer has been rising steadily over the past 40 years, increasing by 3–4% per year for the last 20 years.

Therefore, the identification and use of novel approaches for the prevention and treatment of breast cancer are urgently needed. One such approach is to use retinoids, which are structural and functional analogues of vitamin A, for chemoprevention (1). Retinoids are known to possess antiproliferative, differentiative, and immunomodulatory properties. A growing body of evidence from clinical research supports the concept that retinoids are useful substances in the prevention and treatment of cancer. Retinoids, either alone or in combination with biological response modifiers or chemotherapy, have proved to be effective against skin diseases, acute promyelocytic leukemia, cervical cancer, and other malignancies (2). Furthermore, certain retinoids suppress premalignant oral lesions and prevent the development of secondary primary cancers among patients with head and neck and lung cancers (3, 4). In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens (5). Anzano et al. (6, 7) showed 9-cis RA alone or in combination with tamoxifen or raloxifene to be a very potent inhibitor of mammary carcinogenesis induced by N-nitroso-N-methylurea in Sprague Dawley rats. Tamoxifen and retinyl acetate caused an objective response rate in 39% of 33 patients with advanced breast cancer. In a Phase I/I trial, treatment with tamoxifen plus fenretinide resulted in improvement or disease stabilization in 12 of 15 patients (80%) with no significant adverse effects (8).

The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids is thought to result from their effects on gene expression. These effects are mediated by nuclear receptors, which are ligand-activated transcription factors and members of the steroid hormone receptor superfamily (2).

Two types of receptors have been identified: RAR-α, -β, and -γ and RXR-α, -β, and -γ. Retinoid receptors activate transcription in a ligand-dependent manner by binding to RAREs located in the promoter region of target genes. In DNA binding and transcriptional activation by ligand, retinoid receptors function as heterodimers of RXR and RAR or as RXR homodimers. RXR/RAR heterodimers bind to response elements that generally consist of two directly repeated half-sites of the consensus sequence AGGTCA spaced by 2 or 5 bp (DR2 and DR5 elements; Ref. 9).

One of the target genes of retinoid receptors is the gene encoding RAR-β (10). In its promoter region, a DR5 RA-responsive element named βBARE was identified that mediates RA-induced RAR-β gene expression in many different cell types (11). The RAR-β gene is expressed primarily in normal epithelial tissue, where its expression is up-regulated in response to RA treatment.

Altered nuclear receptor activities are known to be associated with carcinogenesis. The involvement of RAR-β in cancer development was originally suggested by the finding that it was integrated by hepatitis B virus in human hepatoma (12). Recently, it was found that RAR-β was not expressed in a number of malignant tumors, including lung carcinoma, squamous cell carcinoma of the head and neck, and breast cancer cell lines (13-16). Compared with normal tissue, a decrease in RAR-β mRNA levels was also observed in squamous cell carcinoma of the head and neck region (17) and in human epidermoid lung cancer cells (18). All of these findings support the concept that the specific loss of RAR-β expression may be an important event in...
tumorigenesis. This concept is supported by the observation that introduction of RAR-β into RA-insensitive cell lines restored RA sensitivity in breast cancer cell lines (19).

RAR-β is encoded by a gene located at chromosome 3p24. In breast cancer, there is a known high frequency of LOH at chromosome 3p24. Recently, Deng et al. (20) reported that LOH in normal terminal ductal-lobular units adjacent to the tumor was seen in 6 of the 10 cases with confirmed LOH at 3p24 in the carcinoma. Terminal ductal-lobular units taken from tissue samples obtained at a certain distance from the tumor showed no LOH at 3p24. They speculate that LOH in the normal adjacent lobules may define a localized predisposed region from which the cancer arises.

This study was designed to determine whether abnormalities exist in the expression of retinoid receptors in surgical specimens from patients with breast cancer. To investigate whether mutation of βRARE is responsible for loss of RAR-β expression in tumor specimens, the βRARE region was sequenced.

Materials and Methods

Preparation of Digoxigenin-labeled RNA Probes. We used 1.7-kb RAR-α, 1.4-kb RAR-β, and 2-kb RAR-γ cDNA fragments. All templates were obtained from Prof. Pierre Chambon (IGBMC ILLKIRCH Cedex, Strasbourg, France). The RAR cDNAs were subcloned into pBSK (RAR-α, RAR-β) and pBSM13 (RAR-γ) plasmids in either orientation with respect to the T7 and the T3 promoter sites to transcribe antisense and sense (negative control) RNA probes. The plasmids were linearized by restriction enzyme digestion and purified from agarose gel electrophoresis bands using the Wizard PCR Preps DNA purification system (Promega, Madison, WI). For the transcription, 1 μg of linearized DNA was incubated at 37°C for 2 h in a solution containing transcription buffer, digoxigenin-RNA labeling mixture (Boehringer Mannheim, Mannheim, Germany), RNase inhibitor, and T3 or T7 RNA polymerase (21). The reaction was stopped by digesting the DNA template with RNase-free DNase, and RNA probes were precipitated overnight at −70°C with LiCl and 70% ice-cold ethanol. Then the probes were pelleted by centrifugation at 12,000 × g at 4°C and washed again with 70% ethanol. The probes were dried under vacuum, dissolved in 100 μl of diethylpyrocarbonate-treated water with 1 μl of RNase inhibitor, and purified with NickSpin Columns (Pharmacia Biotech, Uppsala, Sweden). To check the concentration and the digoxigenin-labeling of the probes, a Northern blot and a Dot blot were performed.

In Situ Hybridization. Formalin-fixed, paraffin-embedded histological sections stained with H&E were reviewed to identify slides containing invasive ductal breast carcinoma: 7 of 12 specimens proved to be estrogen receptor positive, and 5 of 12 specimens were estrogen receptor negative. In two specimens, no receptor studies were performed. All cancers were stage pT1 or pT2. We used a method recently described by Xu et al. (17): 2-μm sections were deparaffinized with xylene, rehydrated, air dried, and incubated for 20 min with proteinase K at 37°C for deproteinization. After being washed with TBS buffer, the slides were covered with 40 μl of hybridization solution containing 50% formamide, 2× SSC, 10% dextran sulfate, 100 μg/ml of DNA from salmon testes, 0.02% SDS, 19% diethylpyrocarbonate-treated water, and 1% RNA probe (20 ng). Slides were covered with coverslips and incubated at 80°C for 4 h in closed humid containers. The coverslips were then removed in 2× SSC, and the slides were incubated three times for 15 min each time in 50% formamide/1× SSC at 55°C and twice for 10 min each time in 1× SSC at room temperature. After being washed with TBS buffer, slides were blocked for 15 min with 100 μl/slide in buffer 1 containing 0.5% blocking reagent and 10% FCS. For immunodetection of the in situ hybridization signal, the slides were incubated for 1 h with sheep antidigoxigenin antibodies (1 μl/250 μl in buffer 1 [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) containing 0.5% blocking reagent and 10% FCS) and washed five times with TBS buffer. The color reaction was developed by incubating the slides in a chromogen solution (4159 µM of nitroblue tetrazolium chloride and 35 µl of X-phosphate solution in 10 ml of buffer 3 [0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5]; Ref. 22). The color reaction was stopped by washing the slides with water, and the slides were mounted with a cover glass in Kaiser's glycerin gelatin (Merck, Darmstadt, Germany).

Isolation of DNA from Paraffin-embedded Samples. Breast cancer tissue from above mentioned patients was microdissected into 20–40-μm sections (23). Paraffin was extracted by incubating the tissue samples in xylene for 30 min, centrifuging, and removing the supernatant. Ethanol in various concentrations (absolute, 90%, and 70%) was added three times and removed by centrifugation. The samples were dried under vacuum and incubated overnight at 37°C in a digestion buffer containing 200 μg/ml proteinase K.

PCR. To amplify the region containing the βRARE we used the following primer pairs: forward primer, 5' GGA GTT GGT GAT GTG ATC GTA G; and reverse primer, 5' GAT CCC AAG TTC TTC TTC CAA G.

PCR was carried out in a reaction volume of 100 μl containing 100 ng of genomic DNA as template, 1× PCR buffer (Boehringer Mannheim), 200 μM dNTP mix, 300 nM forward primer, 300 nM reverse primer, and 5 units of Taq DNA polymerase (Boehringer Mannheim). After an initial denaturation step at 94°C for 1.5 min, DNA was amplified through 35 cycles consisting of 30 s denaturing at 94°C, 30 s annealing at 55°C, and 45 s extension at 72°C. Fragment length was 324 bp.

DNA Sequencing. PCR fragments were separated from primers using the QIAquick Purification Kit (Qiagen). The purified DNA was subjected to cycle sequencing using an automated fluorescence-based cycle sequencer (LI-COR 4200). Both DNA strands were sequenced.

Results

Expression of RAR-α and RAR-γ in Breast Cancer and Normal Breast Tissue. Expression of the mRNAs for RAR-α and RAR-γ was analyzed by nonradioactive in situ hybridization, which resulted in a dark purple color in the cytoplasm, where mRNA is expected to be localized. Staining intensity was somewhat heterogeneous among the cells in a given specimen, with some cells expressing higher amounts of receptor mRNA than others. The sense probes of the two RARs did not bind to adjacent sections, indicating that hybridization of the antisense was specific. Analysis of the 14 breast cancer cases probed for RAR-α and RAR-γ is summarized in Table 1. Of tumor specimens and tissue distant from the lesion, 100% were positive for RAR-α. mRNA for RAR-γ was detected in 100% of normal tissue, whereas in the tumor it was found in only 11 of 14 (78%) cases analyzed. There was no apparent relationship between estrogen receptor positivity and RAR-α or RAR-γ expression.

Expression of RAR-β in Cancerous Tissue, Morphologically Normal Adjacent Tissue, and Normal Breast Tissue Distant from the Cancer. Performing in situ hybridization in 13 of 14 cases, we were not able to detect mRNA for RAR-β in any breast cancer specimens we analyzed (Fig. 1A). Surprisingly, in none of these 13 cases were we able to find RAR-β transcripts in the morphologically normal appearing breast tissue adjacent to the tumor (Fig. 1A). To study whether the loss of RAR-β expression is a general feature of normal adult breast tissue, we analyzed morphologically normal specimens from the same patients distant from the tumorous lesion and found RAR-β expressed in all cases (Fig. 1C). In one case, the tumor and the adjacent morphologically normal appearing breast tissue stained positive for RAR-β transcripts. The sense probe of RAR-β failed to hybridize to any of the sections (Fig. 1B and D).

Sequencing of the βRARE in DNA Extracted from Tumor Tissue. Loss of expression of the RAR-β gene in the breast cancer tissue could result from mutations in βRARE. RARE characterization was performed by DNA double-strand sequencing analysis. In all 14

<table>
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<th>No. of specimens/total (%)</th>
<th>Breast tissue</th>
<th>Expression of RAR-α</th>
<th>Expression of RAR-β</th>
<th>Expression of RAR-γ</th>
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<td>14/14 (100)</td>
<td>14/14 (100)</td>
<td>14/14 (100)</td>
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<tr>
<td>Tumor</td>
<td>14/14 (100)</td>
<td>1/14 (7)</td>
<td>11/14 (78)</td>
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Table 1 Comparison of expression of RARs in breast carcinoma and nonneoplastic breast tissue distant from the cancerous lesion

RARs were analyzed by in situ hybridization as described in "Materials and Methods." Positive cases were those that stained clearly positive for the particular retinoid receptor compared to the section hybridized with the corresponding sense probe.
breast cancer specimens, as well as in two DNA samples from lymphocytes of two healthy blood donors, we found the same βRARE motif (GGTCACCGAAAGTTCA) without any mutations.

Discussion

This study is the first to use in situ hybridization for the analysis of the mRNAs for RARs in surgical specimens of breast cancer tissue in addition to normal breast tissue distant from and adjacent to breast cancer. We demonstrated here that RAR-β expression is absent in 13 of 14 breast cancer specimens, as well as in the morphologically normal tissue adjacent to the tumor. RAR-β is, however, expressed in all cases of normal breast tissue distant from the tumor. RAR-α was expressed in all cases, whereas RAR-γ transcripts could be found in all normal breast tissues but were detectable in only 78% of tumors investigated.

Concerning expression of RAR-α, our results are compatible with immunohistochemical data (24). In agreement with the results of some authors (24) and in contrast to reports of others (25) who compared estrogen receptor-positive and estrogen receptor-negative cell lines, we did not find a correlation between estrogen receptor and expression of RAR-α transcripts.

Previous studies have shown that RAR-α and RAR-γ mRNA are expressed in most breast cancer cell lines, whereas RAR-β transcripts could not be found (15, 19).

There is much evidence to show that loss of RAR-β expression is an early event in breast carcinogenesis. Swisshelm et al. (14) demonstrated that RAR-β expression increases in senescing normal breast cells but not in established tumor cell lines. The RAR-β gene is located at chromosome 3p24 (9). LOH of chromosome 3p24 is a common event in breast cancer (20). LOH is confined to cancerous lesions and to the normal tissue near the cancer, and this may suggest that the entire mammary gland is not affected. Because regions of LOH are thought to contain recessive genes relevant to malignancy, LOH in the normal adjacent lobules may define a localized predisposed region from which the cancer arises. Conceivably, this predisposed region may be present prior to mammary gland differentiation in the form of single stem cells from localized regions of the mammary gland (26).

Although decreased expression of RAR-β appears to be a common event in breast carcinogenesis, the underlying molecular mechanism is unclear. The RAR-β gene promoter includes a RARE that can be activated by RXR/RAR heterodimers. In breast cancer cell lines, RA is not able to induce RAR-β gene expression; tumor cells can trans-activate an exogenous βRARE (14). These facts support the concept that the endogenous receptor contains point mutations. In this study, we were not able to detect any mutations within the βRARE promoter. Possibly, negative regulation of RAR-β in tumor cells is due to down-regulation of transcription, directed by other regions of the promoter in addition to the RARE, implicating trans-elements. In HeLa cells, it has been shown that communication between the upstream elements and the βRARE seems to be disturbed (27). Loss of transcription factors that induce RAR-β transcription could be another reason for the complete loss of this receptor in the tumor.
cervical cancer cells, RAR-α is the major subtype for induction of RA-dependent RAR-β gene expression (28). In all of our breast cancer specimens, RAR-α transcripts were detected at a very high level. In our study, we could not rule out the possibility that changes of RA metabolism, presence of coactivators or corepressors of tumor cells, or mutations in the whole promoter region of the RAR-β gene are the molecular mechanism underlying the loss of RAR-β gene expression.

The involvement of retinoid receptors in the regulation of gene expression and suppression of growth of breast cancer cells has been demonstrated (Ref. 2 and references therein). In vitro experiments have demonstrated that RAR-β mediates retinoid action in breast cancer cells by promoting apoptosis, and loss of RAR-β may contribute to the tumorigenicity of human mammary epithelial cells (19, 29). Recent evidence specifically implicated RAR-β in tumor suppression in vivo; transfection of a human epithodermoid lung cancer in vitro with an RAR-β expression vector resulted in decreased tumorigenicity (18). In addition, transgenic mice expressing antisense RAR-β2 developed carcinomas 14–18 months after birth (30).

Xu et al. (17) recently demonstrated abnormalities in the expression of RAR-β in malignant head and neck tissues and in non-small-cell lung cancer (31). The same authors also demonstrated that after patients were treated with oral retinoids, RAR-β expression increased in premalignant head and neck cancer lesions and these lesions regressed (32).

Although we examined a small number of specimens, we can state that loss of RAR-β is a general event involved in the tumorigenesis of breast cancer. The most important question, however, is whether retinoid chemoprevention is effective against breast cancer and possibly other cancers. If this is the case, why is the endogenous production of RA not sufficient? We have an example in acute promyelocytic leukemia, where fusion of RAR-α and a promyelocytic leukemia gene is fundamental to disease pathogenesis. In this case, treatment with RA induces complete remission, providing an example of antineoplastic therapy directly targeting the underlying molecular mechanism (33).

Very recently, Minna and Mangelsdorf (34) stated that this example should encourage us to test whether supraphysiological levels of the appropriate exogenous retinoids can overcome diminished RA-induced apoptosis, promoting protein in Greem, B. Baust, C. and Cancer Res., 55: 2135–2139, 1995.


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