Retinoic acid receptors $\alpha$, $\beta$ and $\gamma$, and cellular retinol binding protein-I expression in breast fibrocystic disease and cancer

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Abstract

Retinoids seem to act as agents of chemoprevention and differentiation in breast diseases. Their action is mediated by nuclear receptors, retinoic acid receptors (RAR$\alpha$, RAR$\beta$, RAR$\gamma$) and retinoid X receptors (RXR$\alpha$, RXR$\beta$, RXR$\gamma$) and modulated by cellular retinol binding proteins (CRBP). There are few published data on CRBP expression. In this study, we evaluated the expression of RAR$\alpha$, $\beta$ and $\gamma$ and CRBP type I (CRBP-I) gene expression in fibrocystic disease (FD) and in breast cancer (BC), studying 14 FD and 20 BC surgical samples by reverse transcription (RT)-PCR. We also evaluated mRNA concentrations in cancer samples by a semiquantitative PCR method, co-amplifying RAR$\alpha$, RAR$\beta$ and CRBP-I genes with an unrelated gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as internal control. All benign and malignant breast tissues expressed RAR$\alpha$, $\beta$ and $\gamma$, and CRBP-I mRNAs. A greater concentration of RAR$\beta$ mRNA was detected in cancer tissues with lower oestrogen and progesterone receptor concentrations, whereas RAR$\alpha$ was detected in variable concentrations that were not related to those of steroid receptors. The CRBP-I concentration was similar in all samples studied. We demonstrated that all three RARs and CRBP-I transcripts are expressed in FD, and that RAR$\beta$, RAR$\gamma$ and CRBP-I mRNAs also are present in BC tissues. This indicates that both malignant and benign breast tissues may be target for retinoids, justifying the use of natural and synthetic vitamin A derivatives in the chemoprevention of breast disease.

Introduction

Retinoids, including vitamin A (retinol) and the active compound retinoic acid, have been shown to be effective inhibitors of chemical carcinogenesis in epithelial tumours such as those of the skin, lung, breast, prostate, cervix, bladder and oesophagus (1). Retinoic acid is the substance of choice in trials on the chemotheraphy of prevention and differentiation of neoplastic diseases, because it is utilized immediately, without any appreciable hepatic storage (2, 3). New retinoid compounds, with modifications to the basic structure, show enhanced target organ specificity and increased inhibitory activity with reduced systemic toxicity (4). Current clinical studies with retinoic acid derivatives are aimed at prevention of the recurrence of breast cancer (BC) in humans (5). Retinoids act as ligands for a number of nuclear receptor proteins, called retinoic acid (RAR$\alpha$, $\beta$ and $\gamma$) and retinoid X (RXR$\alpha$, $\beta$ and $\gamma$) receptors, belonging to the superfamiliy of nuclear receptors that includes thyroid hormone, vitamin D and steroid hormone receptors (6–8). Nuclear receptors bind the specific DNA-responsive element through heterodimer (RAR–RXR) or homodimer formations (9, 10). Even if there is a functional redundancy among the different RARs, various receptor subtypes possess distinct functions regulating the expression of different genes (10–14). In addition, retinoids bind another family of proteins probably involved in their intracellular binding, transport and metabolism, including the cellular retinol binding protein type I (CRBP-I) and type II (CRBP-II) and two cellular retinoic acid binding proteins (CRABP) (6).

Evidence that specific nuclear receptors mediate the action of retinoids on transcription and that binding proteins modulate their intracellular metabolism raises the question of whether benign and malignant breast tissues express retinoid receptors and binding proteins. To elucidate the mechanism accounting for the inhibitory effect of retinoids on the breast, several investigators have utilized both in vitro and in vivo model systems, but little is known about the expression and function of RARs in tissues from BC and benign breast diseases. RAR and RXR gene expression have been
Material and methods

Origin of tissues

Breast tissue samples from 34 patients with FD or with BC were provided by the tissue core facility of the National Cancer Institute ‘G. Pascale’, Naples. Fresh tissue was obtained directly from the operating room, frozen immediately in liquid nitrogen and kept at −70°C until required for extraction of total RNA. We studied 14 tissue samples from women of mean age 45 years (s.d. 12, range 27–63 years) with histological diagnosis of FD, and 20 samples from women of mean age 49 years (s.d. 10.5, range 29–67 years) with BC. Clinical and pathological data of the BC patients studied are shown in Table 1. The grading of invasive carcinoma of the breast was based on criteria from Elston (18).

RT-PCR analysis

Total RNA extracted from breast fibrocystic and carcinoma tissues was subjected to reverse transcription and amplified by PCR using RARα, β and γ, and CRBP-I specific primers. Total RNA was extracted from tissue samples by the guanidine thiocyanate method (19). Reverse transcription was performed as described previously (16), using 400 U Superscript RNase H reverse transcriptase (Gibco BRL, Milan, Italy). As template for the PCR reaction, we used 400 ng (RARs) cDNA in a total volume of 25 μl Taq-polymerase buffer (Perkin Elmer, Milan, Italy) containing 5 mmol/l dNTPs, 50 ng each primer, and 2 U Taq DNA polymerase (Perkin Elmer). PCR conditions were as described previously and the reaction consisted of 32 cycles of amplification (16). Amplified product reproducibility was confirmed by two PCR rounds. To evaluate any variability in the expression of RARs and CRBP-I in individual tissue samples, a semiquantitative PCR was performed in which RARα and β, and CRBP-I genes were amplified with an unrelated sequence as internal control. When quantitative RT-PCR was performed, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified (22 cycles) simultaneously, its specific primers being added to the PCR reaction after the first 10 cycles. The number of cycles was chosen in the middle of the exponential phase of the reaction, separately for each tissue. To establish the number of cycles, GAPDH and RARs were amplified at 20, 25, 35 and 40 PCR cycles. Figure 1 shows an example of co-amplification of GAPDH (876 bp) and RARβ (400 bp) from 20 up to 40 cycles. All RARs amplimers were still in the linear phase of the PCR reaction at 35 cycles. The ratio of intensity of the two bands was used as an indication of the relative abundance of the two templates in the samples. We used as primers (supplied by CEINGE Oligosynthesis Service, Naples, Italy): RARα sense (5’ TGCTGGAGGGCGCTAAAAAGTGC 3’) and antisense (5’ TGTCCAAGGAATGGCTGCC 3’); RARβ sense (5’ TGGCCTTGGAATGGATGAC 3’) and antisense (5’ TGAAGTCCCAGCAGTGG 3’); RARγ sense (5’ GGCTAACAAGTGCAATCACAAGA 3’) and antisense (5’ AGGAGGTCCCTCCGAGCTACT 3’); GAPDH sense (5’ GACCCCTTATGTACAGTATG 3’) and antisense (5’ GTCGACCCCTCTTGTGTAGGCC 3’); CRBP-I sense (5’ GTGCTGGGAAGATGGTG 3’) (20); CRBP-I antisense (5’ AGCTGGTCGAGCCCT 3’) (21). PCR products were separated on a 1.2% agarose gel containing ethidium bromide using φX174/Hae III (Stratagene, M-Medical, Florence, Italy) or 100 bp DNA ladder (Gibco BRL, Milan, Italy) as size marker.

Results

RT-PCR, using appropriate primers, gave rise to products of the expected size for RARα, β and γ ligand binding domain regions in all samples investigated. Examples are shown in the Figures, as follows. Figure 2 shows the RARα (495 bp) and RARβ (400 bp) transcripts obtained in representative BC and FD tissues from the 34 samples examined. Figure 3 shows RARγ (736 bp) transcripts in two representative BC tissues and four representative BC tissues; in two of the latter, RARγ mRNA of 736 bp was detected along with two PCR products of 600 and 350 bp. Figures 4 and 5 show, respectively, the RARα and RARβ transcripts co-amplified with the GAPDH (876 bp) gene, using semiquantitative RT-PCR, in 18 BC tissues (from patients 1–18 in Table 1).
Figure 2 Expression of RAR\(_a\) and \(\beta\) in representative samples from breast fibrocystic disease and cancer tissues detected by PCR. PCR products were separated on a 1.2\% agarose gel. Lane 1: \(\phi\)X174/Hae III DNA size marker; lanes 2 and 3: 495 bp products of RAR\(_a\) detected in fibrocystic and cancer tissues, respectively; lanes 4 and 5: 400 bp products of RAR\(\beta\) in breast fibrocystic and cancer samples, respectively.

Figure 3 Expression of RAR\(_g\) in representative samples from breast fibrocystic disease and cancer tissues detected by PCR. PCR products were separated on a 1.2\% agarose gel. Lane 1: \(\phi\)X174/Hae III DNA size marker; lanes 2 and 3: 736 bp products of RAR\(_g\) in two fibrocystic tissues; lanes 4–7: 736 bp products of RAR\(_g\) in four cancer tissues. Lanes 4 and 5 also show 600 and 350 bp PCR products co-amplified by RAR\(_g\) specific primers.

Figure 4 Co-amplification by semiquantitative RT-PCR of RAR\(_a\) (495 bp) and GAPDH (876 bp) in 18 breast cancer (lanes 2–19) and two fibrocystic disease (lanes 20 and 21) tissue samples. Lane 1: 100 bp DNA ladder size marker. The intensity of the RAR\(_a\) transcripts is variable, indicating a different pattern of expression between BC samples.

Figure 5 Co-amplification by semiquantitative RT-PCR of RAR\(_\beta\) (400 bp) and GAPDH (876 bp) in 18 breast cancer (lanes 2–19) and two fibrocystic disease (lanes 20 and 21) tissue samples. Lane 1: 100 bp DNA ladder size marker. RAR\(\beta\) transcripts were more abundant in BC tissue samples that also had low contents of oestrogen receptor (see Table 1 and text).

Table 1 Clinical, pathological and steroid receptor data, and relative expression of RARs and CRBP-I genes in 20 cancer tissues sampled.

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<th>Age (years)</th>
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<th>Tumour grade</th>
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<th>PR (fmol/mg)</th>
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ER, oestrogen receptor; PR, progesterone receptor; IC, invasive carcinoma; p, present (level of expression is indicated in parentheses); na, not assayed.
and two FD tissues. In BC tissues samples, the expression of RARα and RARβ was variable. In all BC and FD samples, a 340 bp product was amplified that corresponded to the expected size of the CRBP-I transcript. Figure 6 shows the CRBP-I transcripts obtained by semiquantitative RT-PCR in 18 BC and two representative FD tissues, showing a uniform level of expression between samples. Table 1 shows the levels of expression of RARα, RARβ and CRBP-I genes in individual cancer samples, together with the associated concentrations of their oestrogen and progesterone receptors and tumour grades; the concentrations of RARβ mRNA were greater in samples having very low amounts of these two receptors (Table 1 and Fig. 5).

**Discussion**

Using RT-PCR, we have demonstrated that RARα, β and γ, and CRBP-I transcripts are present in all breast tissue samples obtained from patients after surgery for either FD or BC. To date there have been no reports concerning expression of RARs in breast tissues from FD, whereas RARα and β transcripts have been found previously in normal breast tissue (15). In primary breast tumour samples, RARα mRNA has been detected only by Northern blot analysis (17). Our ability to detect all three RAR mRNAs in breast cancer tissues may be due to our use of the more sensitive technique of PCR; our RT-PCR technique enabled us to obtain immediate and reliable results, even with very small amounts of tissue. We can exclude the possibility that the PCR products were derived from contaminating genomic DNA, because the presence of any introns in the domains that we amplified would have resulted in larger PCR products. In two BC samples examined, 600 and 350 bp products were co-amplified with the expected RARγ transcript, supposedly as a result of an alternative splicing of the RARγ gene; however, an amplification artefact cannot be excluded.

Several pieces of evidence suggest a mutual regulation between steroids and retinoids in breast cancer cell lines and tissues. RARα has been reported to be more highly expressed in oestrogen-receptor-positive cell lines and to be positively correlated with oestrogen receptor transcript levels in 93% of breast cancer tissue samples, whereas RARβ expression was more abundant in cancer tissues with lower levels of the oestrogen and progesterone receptors (17, 22). In addition, oestradiol has been reported to induce RARα in human breast cells (17). RARγ expression has been reported in all cell lines studied, independent of oestrogen receptor status (22). The pattern of expression of RARβ in our BC tissues confirms previous findings in oestrogen-receptor-negative cell lines (17, 22), and RARα mRNA appeared not to be related to levels of either oestrogen receptor or progesterone receptor. There are no previously published data concerning the expression of CRBP-I in breast diseases, but we have now demonstrated that CRBP-I mRNA is present in both benign and malignant breast tissue samples, with similar patterns of expression in all breast samples studied. This binding protein may contribute to the maintenance of physiological concentrations of intracellular retinoids and to the control of retinoic acid metabolism. It has been speculated that the tissue expression of CRBP-I is related to retinoic acid requirement and metabolism, and it has been demonstrated that retinoic acid-induced inhibition of cell growth depends on intracellular retinoic acid metabolism, modulated by CRBP-I (6, 23). Our findings concerning CRBP-I in breast cancer tissue samples are thus of interest.

Retinoids are considered to be agents of chemoprevention and differentiation in several proliferative diseases, including benign and malignant breast diseases (5, 24–26). Knowledge of how retinoids interrupt proliferative processes pharmacologically or through diet, and how they induce differentiation and programmed cell death, will probably lead to new treatment strategies, especially in the prevention of breast cancer. In experimental studies using mice lacking RARs, RXRs, or both, no alteration of the breast gland was reported, but degeneration of the testicular germinal epithelium, and keratinizing squamous metaplasia of the seminal vesicle and prostate, as seen in vitamin A-deficient animals, have been described (11–13). However, several studies in breast cancer cell lines have elucidated the function of retinoids and their receptors in the regulation of cell growth and differentiation, the alteration of carcinogen metabolism, oncogene expression, apoptosis and the steroid/retinoid receptor relationship to growth factors (25–27). Retinoids may exert their antiproliferative effects on breast cells by transactivating genes via retinoic acid response elements or by direct action against activator protein 1 (AP1) (14, 28–30). There is evidence that RARβ is essential for retinoic acid-induced inhibition of growth and apoptosis in human breast cancer cells (31), and some retinoic acid analogues can act through a direct anti-AP1 effect, inducing cytostatic effect or G0/G1 arrest and apoptosis (14, 28–30, 32).

With the present study, we have added more information about the role of retinoids and their
receptors in the physiopathology of the human breast. Our data on the presence of RARs and CRBP-I transcripts in FD and BC indicate that they are targets for retinoids. Therefore, all three RARs can mediate the putative chemopreventive effect of retinoids on the breast. The qualitative and quantitative differences of RARs receptor subtypes between different breast cancer samples suggest that a complex mechanism, partially related to steroid hormone responsiveness, is involved in the regulation of RAR expression in breast cancer. We have also provided evidence that cancer tissues express CRBP-I without differences in concentration between individual samples.

In conclusion, our findings suggest that benign and malignant breast tissues require retinoids and are responsive to them, which justifies the use of natural and synthetic vitamin A derivatives in the chemoprevention of breast diseases (5, 23–25).

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