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5′Deiodinase in two breast cancer cell lines: effect of triiodothyronine, isoproterenol and retinoids

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Abstract

Thyroid hormones participate in the regulation of growth, development and energy expenditure of vertebrates. Type I (D1) and type II 5′deiodinases catalyze the peripheral conversion of the thyroid prohormone thyroxine to the active form triiodothyronine (T3). D1 is expressed in organs like liver, thyroid, and lactating mammary gland. This enzyme is regulated in an organ-specific manner by a wide number of factors like carbohydrates, T3, thyrotropin, and catecholamines. However, it has been shown that in several types of cancer the expression of D1 is reduced, lost, or regulated by different components. In the present work we describe the expression and regulation of 5′deiodinases in two breast cancer cell lines: MCF-7 (ovarian hormone-dependent) and MDA-MB-231 (ovarian hormone-independent). Our results showed that MCF-7 cells expressed D1 activity ($\approx$ 10 pmol I^-/mg protein per h), which was stimulated only by retinoic acid treatments, but not by T3 or the β-adrenergic agonist isoproterenol. In MDA-MB-231 cells, deiodinase activity was not detected in control conditions nor under any of these treatments. These results support the notion that D1 expression could represent a sensitive differentiation marker.

Keywords: Breast cancer cell lines; Deiodination; Retinoids; Thyroid hormones; β-Adrenergic agonists

1. Introduction

Thyroid hormones (TH) participate in the regulation of energy expenditure as well as in the development and differentiation of various vertebrate tissues. The enzymatic process called deiodination modulates the biological action of TH at peripheral level, and plays a key role in determining the intracellular levels of active or inactive thyronines (Köhrle, 2000). Prohormone thyroxine (T4), the main secretory product of the thyroid gland, becomes an active (Triiodothyronine; T3) or inactive hormone (reverse T3; rT3) under deiodination of its outer (5′) or inner ring (5), respectively. Based on their kinetic and molecular characterization three distinct deiodinase enzymes have been identified: type I (D1), type II (D2) and type III. D1 and D2 catalyze the activating pathway of TH. D1 provides most of the circulating T3, and it is predominantly expressed in liver, kidney and thyroid gland, but is also detected in heart, pituitary and lactating mammary gland (Aceves and Valverde-R, 1989; Köhrle, 2000; Bianco et al., 2002). TH play an important role in the growth and development of mammary gland (Vonderhaar and Greko, 1979). We previously reported that rat mammary gland expresses both D1 and D2 (Aceves and Valverde-R, 1989; Aceves et al., 1995), but in lactation, D1 increases appreciably in the alveolar epithelium (Aceves et al., 1995), and its expression is regulated by the suckling stimulus, through β-adrenergic stimulation (Aceves et al., 1999a,b). These findings suggest that D1 is a local supply of T3 in mammary gland supporting high-energy expenditure associated to proliferation, differentiation, and milk production (Aceves et al., 1995, 1999a). Based on milk analysis, it has been reported that human lactating mammary gland expresses both D1 and D2 enzymes (Slebodzinski et al.,

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1993), although their physiological regulation remains to be established.

Evidence that abnormal thyroid function is related to an increased risk of breast cancer remains controversial. The most accepted facts are that slight hypo- or hyperthyroidism is associated with an increased incidence of induced mammary cancers, or to a reduction in cancer size, respectively. The mechanisms by which thyronines might exert these effects are not clear (Guernsey and Fisher, 1990; Jull and Huggins, 1960; Milmore et al., 1982). Recent studies in our laboratory using breast cancer samples have shown a differential deiodinase expression related to the cellular origin of the cancer. That is, ductal cancers express both D1 and D2, whereas lobular cancers express predominantly D1. Moreover, we have found that, regardless of the cellular origin, cancer progression has an indirect relationship to D1 expression (Gallardo de la et al., 2000; Gallardo de la O and Aceves, unpublished data). In addition, the expression of D1 enzyme has been corroborated by Macejova et al. (2001) and by us (Aceves et al., 2002) in rat mammary carcinomas induced by *N*-methyl-*N*-nitrosourea (MNU). D1 mRNA expression was higher in MNU-tumors arising in the first 2–4 months than those arising after 6 months (Aceves et al., 2002). These data are in agreement with the proposal that D1 expression might represent a differentiation marker in carcinoma cells (Schreck et al., 1994). It has been shown that in several cancers, the expression of this enzyme is reduced, lost, or regulated by different components (Köhrle, 2000; Pachucki et al., 2001). In differentiated thyroid carcinoma cells, D1 expression is reduced and refractory to its physiological stimuli (T3 and thyrotropin; TSH), but becomes responsive to retinoic acid (RA). Additionally, It has been reported that RA not only affects D1 expression in these type of cells and tumors but also others several thyroid-relevant genes, such as the sodium/iodide symporter (NIS), intercellular adhesion molecule-1 and E-Cadherin (Schmutzler and Köhrle, 2000). In contrast, in totally dedifferentiated thyroid carcinoma cells (anaplastic), D1 expression is undetectable and does not respond to any physiological or RA stimuli (Schmutzler et al., 1996; Schreck et al., 1994).

The present study was designed to characterize the deiodinative type present in two breast cancer cell lines, and to study their response to T3, a β-adrenergic agonist, isoproterenol (ISO), and RA. We reported here that MCF-7 cell line, which preserves several normal epithelium differentiated parameters (estrogen and progesterone receptors, casein synthesis, etc.), expressed D1 activity, which was stimulated by RA but not by T3 or ISO. In contrast, MDA-MB-231, which does not exhibit ovarian hormone receptors or casein expression (Hay et al., 1994; Shao et al., 1995), displayed neither basal deiodinative activity, nor its RA induction or responsiveness.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution and penicillin and streptomycin solution were obtained from GIBCO-BRL (Grand Island, NY). All-*trans* RA and 9-*cis* RA were purchased from Sigma Chemical Co. (St. Louis, MO). Nonradioactive thyronines were obtained from Henning Co. (Berlin, Germany). [^{125}I]rT3 (SA, 1174 μCi/μg) was purchased from New England Nuclear (Boston, MA). Propylthiouracil (PTU) was obtained from US Biochemical Co. (Cleveland, OH) and dithiothreitol (DTT) was obtained from Calbiochem (La Jolla, CA). All other reagents were of the highest purity commercially available.

All-*trans* RA and 9-*cis* RA were dissolved at a concentration of 20 μM in absolute ethanol, stored protected from light at −20 °C and always freshly diluted before use. All manipulations with RA were performed under dim light.

2.2. Cell culture

Human hepatocellular carcinoma cell line HepG2 (ATCC HB-8065) was purchased from ATCC (Rockville, MD). Breast cancer cell lines MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were kindly supplied by Instituto Nacional de Cancerología of México (INCAN). All cell lines were cultured in DMEM supplemented with 10% (v/v) FBS, 3 nM H_2SeO_3, 100 U/ml penicillin and 100 μg/ml streptomycin (basal medium). Cells were incubated in a humidified 90% air, 5% CO_2 atmosphere at 37 °C. For the experiments, cells were seeded at 1.8×10^4 cells per cm^2 in 6-well plates (Corning, NY). When the cells reached 85–90% confluence, the medium was changed as indicated in each experiment. For RA treatments, control cultures received their respective amount of ethanol, the solvent for RA. Concentrations of ethanol never exceeded 0.1% (v/v).

2.3. Enzyme assay

For the enzymatic procedures cell homogenates were prepared by homogenization in ice-cold buffer (10 mM HEPES, pH 7.0, with 0.32 M sucrose, 1.0 mM EDTA, and 10 mM DTT) and stored at −70 °C before use. 5′Deiodinase (5′D) activity was determined by a modification of the release of radiolabeled iodide method (Leonard and Rosenberg, 1980) and standardized for mammary gland (Aceves and Valverde-R, 1989). Assays contained 100–200 μg protein; substrate and cofactor concentrations were optimal for each experimental procedure. To determine the 5′D type present in each

cell line, the enzyme activity was carried out in parallel assays, with and without 1 mM PTU, containing: 2 nM [^{125}I] rT3 and 20 mM DTT. D1 activity assay conditions were as follows: 2 nM [^{125}I] rT3, 0.5 μM nonradiolabeled rT3, and 5 mM DTT. Incubation time was 3 h at 37 °C. The released acid-soluble radioiodide was isolated by chromatography in Dowex 50W-X2 columns. Proteins were measured by the Bradford method (Bio-Rad protein assay, BIO-RAD, Richmond, CA). Results are expressed as picomoles of iodide released per milligram protein per hour (pmol I^-/mg protein per h).

2.4. D1 mRNA expression

D1 mRNA was identified by using a previously standardized semi-quantitative PCR procedure in which an amplicon of the structural protein cyclophilin (Cyc) was simultaneously amplified (Aceves and Rojas-Huidobro, 2001). Briefly, the RT reaction was primed with oligo (dT) and subscripted with 5 μg of total RNA. The PCR reaction was carried out using 1 μl of RT mixture and the following primers: for D1, 377-GTA CCT GAC CTT CAT TTT G (sense), 627-CTG GCT GCT CTG GTT CTG (antisense), whereas for Cyc, 7-AGA CGC CGC TGT CTC TTT TCG (sense), 527-CCA CAC AGT CGG AGA TGG TGA TC (antisense). Amplification was carried out for 40 cycles. Each cycle consisted of melting at 94 °C for 30 s, annealing at 54 °C for 45 s, and an extension at 72 °C for 5 min. As a control, a reaction mixture containing an RNA sample with the appropriate oligonucleotide primers, but without the reverse transcriptase (RT-) was included in every experiment. The sizes of the resultant PCR fragments were 250 bp for D1 and 521 bp for Cyc, and were resolved on a 2% agarose gel and visualized using ethidium bromide. The sizes of the bands were confirmed by a commercial DNA ladder (100 pb DNA ladder, GIBCO-BRL).

2.5. Statistical analysis

Data are expressed as the mean ± S.D. Differences between experimental groups were analyzed using a one-way analysis of variance (ANOVA) Kruskal–Wallis and Mann–Whitney *U*-test or Student's *t*-test. Differences with a $P < 0.05$ were considered statistically significant.

3. Results

3.1. HepG2 cells

Hepatocellular carcinoma cell line, HepG2, has been shown as a suitable model of differentiated liver carcinoma and liver physiology since it retains many of the hepatocyte specific molecular markers (Hay et al., 1994). In previous reports HepG2 cells have been shown to express D1 activity and both T3 and RA are able to stimulate its expression (Schreck et al., 1994; Toyoda et al., 1995). Thus, in the present work HepG2 cells were used as a positive control. The cells expressed D1 activity (44.1 ± 9.7 pmol I^-/mg protein per h) and their expression was stimulated by T3 (68.3 ± 9.6 pmol I^-/mg protein per h) and RA (82.73 ± 11.1 pmol I^-/mg protein per h) treatments but not by ISO (35.06 ± 4.4 pmol I^-/mg protein per h).

3.2. MCF-7 cell line

MCF-7 cells are considered as an epithelial differentiated carcinoma cell line since they conserve several characteristics of epithelial cells such as estrogen and progesterone receptors, and they are able to synthesize casein and NIS (Constantinou et al., 1998; Hay et al., 1994; Kogai et al., 2000). Fig. 1 shows that 5′D activity expressed in MCF-7 cells was sensitive in 85% to PTU inhibition. Fig. 2(A and B) shows the results when these cells were treated with different doses of ISO, a β-adrenergic agonist (6.5, 13 and 130 μM) and T3 (25, 50 nM, and 1 μM). These treatments were applied a single time and maintained for 24 h. None of these treatments affected D1 activity. Moreover, using a single dose of ISO or T3 (13 μM and 50 nM, respectively) and analyzing it at different times (12, 24 and 48 h), we could not find any modification of D1 activity by either of these compounds (Fig. 2C). To analyze whether RA had a stimulatory effect upon MCF-7-D1, we used two isomers of RA (all-*trans* and 9-*cis*). Fig. 3A shows that both RA isomers increased D1 activity, but all-*trans* RA was more effective (10 nM), whereas 9-*cis* RA required 10 times more (100 nM) to induce a similar effect. To evaluate the time course of this effect, the cells were cultured in a medium containing 100 nM of either all-

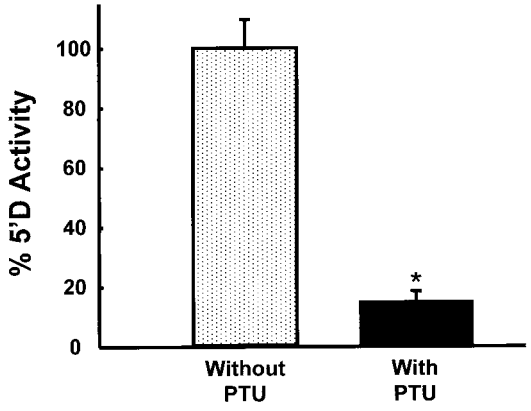
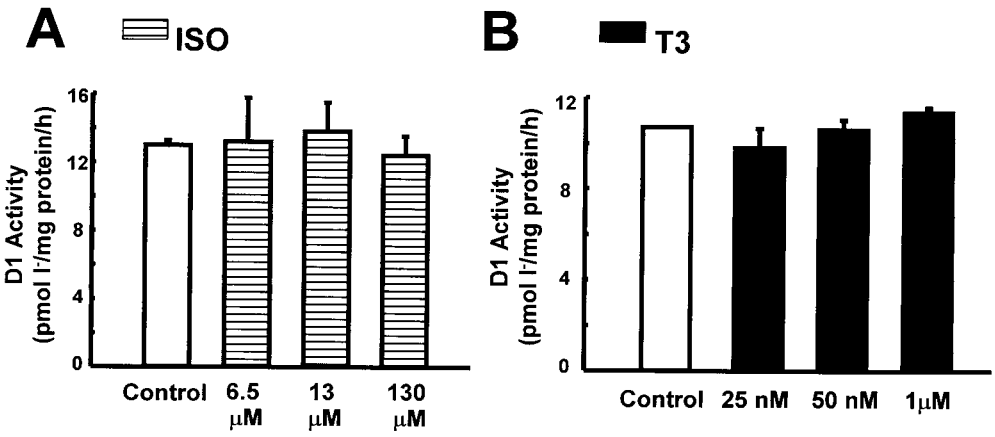


Fig. 1. Effect of 1 mM PTU over 5′D activity in MCF-7 cells. Cells were cultured as described in Section 2. When the cells reached 85–90% confluence, they were harvested. Data were analyzed with Student's *t*-test. *($P > 0.05$). Values represent the mean ± S.D. of three different culture dishes.



C

Treatment	D1 activity (pmol I⁻/mg protein/h)		
	12 h	24 h	48 h
Control	9.14 ± 3.4	8.2 ± 2.9	10.8 ± 3.4
ISO (13 μM)	11.16 ± 5.11	9.68 ± 1.10	10.38 ± 3.08
T3 (50 nM)	9.61 ± 4.48	10.56 ± 4.6	9.07 ± 2.52

Fig. 2. D1 activity in MCF-7 cells treated with ISO and T3. After the cells reached 85–90% confluence, the basal medium was changed and fresh basal medium was added with different concentrations of ISO or T3; cells were maintained for 24 h (A and B). Values represent mean ± S.D. obtained from two culture dishes. In a second tandem of experiments cells were incubated with ISO (13 μM) or T3 (50 nM) for different times (12, 24 and 48 h) (C). Values represent mean ± S.D. obtained from three different experiments. Statistical analysis was made with a one-way ANOVA Kruskal–Wallis test; no significant differences were found ($P > 0.05$).

trans or 9-*cis* RA (Fig. 3B) during 12, 24 and 48 h. All-*trans* RA induced a significant increase in D1 activity after the first 12 h. After 24 h of RA treatment, both isomers increased D1 activity in a significant manner. The maximum increment in D1 activity was detected at 48 h for all-*trans* RA treatment; whereas for the same time interval, D1 activity with 9-*cis* RA treatment seemed to decay. All the results shown here were obtained using DMEM supplemented with 10% FBS. In our first experiments we used a medium containing 10% charcoal-treated FBS (free of thyronines, steroids and RA) and we obtained the same cell responsiveness to treatments; therefore, we decided to use only whole FBS.

3.3. MDA-MDB-231 cells

This cell line does not express several breast epithelial markers such as estrogen and progesterone receptors, casein nor NIS, so they may be considered more dedifferentiated than MCF-7 cells. Using identical conditions of cell culture, treatments and enzyme assay, we established a comparison of 5′D activity between MCF-7 and MDA-MB-231 cells. We found that in MDA-MB-231 cells, 5′D activity was not detected (Table 1); and any of the treatments tested (T3, ISO, and RA) did not induce D1 activity (data not shown). To corroborate the presence of D1 mRNA expression in this tumoral cell lines, a semiquantitative RT-PCR was carried out. Fig. 4 shows that only in MCF-7 cells, basal and treated with retinoids, D1 mRNA amplifications were detected. The increase in D1mRNA observed in MCF-7 cells treated with retinoids was in accordance to that obtained for enzymatic activity, suggesting that this RA stimulatory effect involved transcriptional processes.

4. Discussion

In the present study we demonstrated that D1 was present in breast cancer cell line MCF-7 (epithelial differentiated carcinoma) and that it was stimulated by RA, but not by normal mammary D1 enhancers: T3 or ISO. These data agreed with previous findings showing that in several cancers the expression of this enzymatic type lost its physiological regulation and became responsive to RA (Pachucki et al., 2001; Schreck et al., 1994; Schmutzler et al., 1996). Moreover, the data showing that dedifferentiated MDA-MB-123 cell line

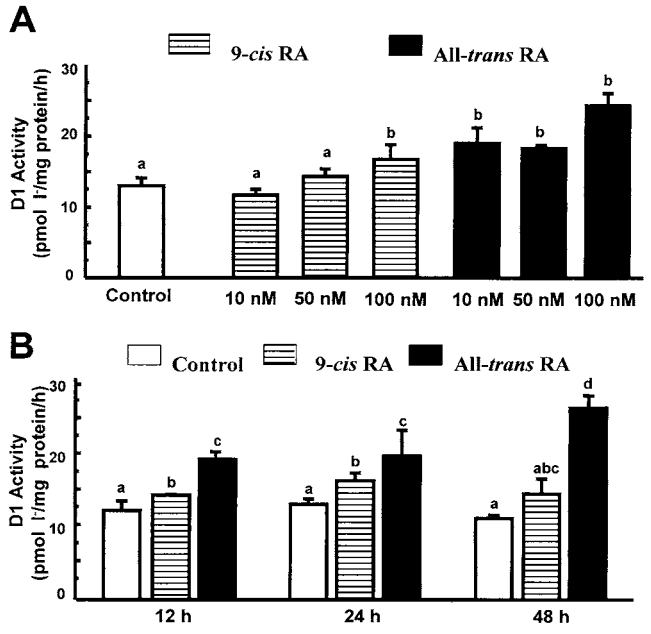


Fig. 3. Effect of RA isomers on D1 activity in MCF-7 cells. (A) Dose-dependent changes in D1 activity. After the cells reached 85–90% confluence, the basal medium was changed and fresh basal medium was added with different concentrations of 9-*cis* RA or all-*trans* RA; cells were maintained for 24 h. Values represent mean ± S.D. obtained from two culture dishes. (B) Time course of RA-induced D1 activity. In a second experiment cells were incubated with all-*trans* (100 nM) and 9-*cis* RA (100 nM) for different times (12, 24 and 48 h). Values represent mean ± S.D. obtained from three culture dishes. Data in both panels were analyzed with a one-way ANOVA Kruskal–Wallis and Mann–Whitney *U*-tests. Means with different letters indicate significant differences ($P < 0.05$).

did not express any deiodinase activity also supported the notion that the expression of D1 activity could be associated with epithelial differentiation in cancer cells (Schreck et al., 1994; Gallardo de la et al., 2000; Aceves et al., 2002).

Steroid-receptor status of breast cancer cells has been related to their malignancy, progression, and RA responsiveness (Dickson and Lippman, 1995; Liu et al., 1996; Nandi et al., 1995; Thordarson et al., 2001). Negative steroid-receptor breast cancer cells have been considerate, more dedifferentiated and malignant than their positive steroid-receptor counterparts. The mechanism through which cancer cells lost their steroid receptors is not clear and the possible participation of TH in this process has not been explored. Most of the actions of TH, RA, as well as of estrogens, are exerted via members of nuclear receptor superfamily of ligand-inducible transcription factors (Yang et al., 1999; Yen, 2001). Recent evidence has demonstrated the presence of a significant cross-talk between TH, RA, and estrogens (Es) in the stimulation of growth and differentiation of both normal and pathologic mammary epithelium (Dinda et al., 2002; Lee et al., 1995; Shao et al., 1995). In the specific case of D1, it has been demonstrated that the promoter of human D1 gene contains two complex combined thyroid hormone and RA responsive elements (TRE and RARE, respectively) (Köhrle, 2000). In normal cells, however, D1 is stimulated only by T3; whereas in carcinoma cells, and depending on their differentiation condition, D1 looses responsiveness to T3 and becomes sensitive to RA. Moreover, the major dedifferentiation state (anaplastic tumor cells), mostly ovarian-hormone independent, is accompanied by the complete disappearance of D1 expression and its RA induction or responsiveness. These data indicate a complex interaction of T3/RA/Es, and suggest that cancer progression may be associated with alteration in metabolism and/or signaling pathways of these components. It has been shown that both MCF-7 cells and MDA-MB-231 cells are unable to synthesize RA (Mira-Y-Lopez et al., 2000). Furthermore, MDA-MB-231 cells are considered a RA resistant breast cancer cell line since the expression of RARα and RARβ is lower than in MCF-7 cells (Liu et al., 1996). Present results showing that MCF-7–D1 responded to RA, and that all-*trans* RA had a higher effect than 9-*cis* RA, could be explained by the fact that these carcinoma cells express the cellular retinoic acid binding protein II (CRABP-II) (Mira-Y-Lopez et al., 2000). It has been demonstrated that this protein, transporting cytosolic RA (locally synthesized or taken up from the circulation) into the nucleus, also has physical and functional interactions with RAR–RXR heterodimeric receptor, whose affinity is higher to all-*trans* RA rather than to 9-*cis* RA (Delva et al., 1999).

Another interesting finding in the present study was the complete failure, in both cellular types, of a D1 response to adrenergic stimulation. Previous data in our laboratory in lactating rats have shown that mammary D1 is significantly stimulated by β-adrenergic agonists such as norepinephrine (NA) or ISO (Aceves et al.,

Table 1
Comparison of 5′D activity between MCF-7 and MDA-MB-231 cell lines

Cell line	Protein concentration (μg per tube)	[^{125}I] released (%)	PTU inhibition (%)
MCF-7	100	11.6	95
MDA-MB-231	95.4	Undetected	–

Cells were cultured as described in Section 2. When the cells reached 85–90% confluence, they were harvested. 5′Deiodination assay: 2 nM de [^{125}I] rT3, 20 mM DTT, with or without 1 mM PTU. The 5′D activity was determined in both cell lines in the same assay per duplicate.

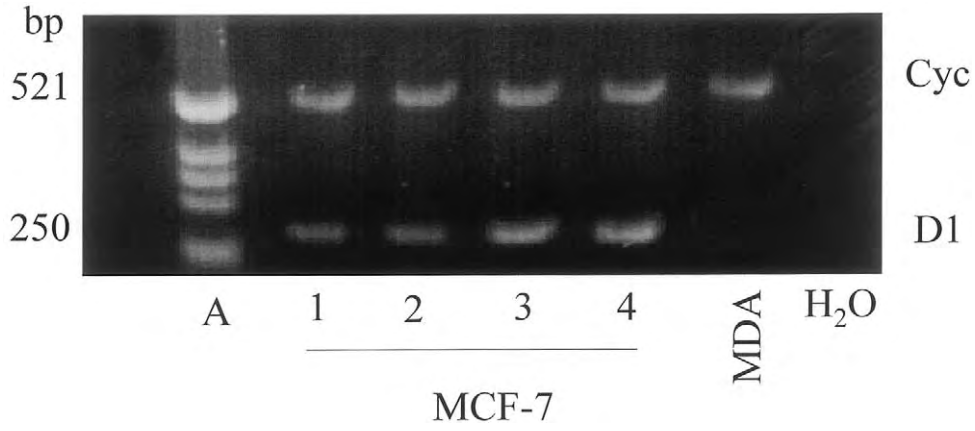


Fig. 4. Comparison of mRNA for deiodinase type 1 (D1) in MCF-7 and MDA-MB-231 cells. Ethidium bromide-stained gel showing RT-PCR products for D1 (250 bp) and cyclophilin (Cyc; 521) after 40 amplification cycles. A, ladder; 1, MCF-7 control; 2, MCF-7 48 h after ethanol treatment; 3, MCF-7 after 48 h 100 nM 9-*cis* RA treatment; 4, MCF-7 after 48 h 100 nM all-*trans* RA treatment; MDA, MDA-MB-231 control; H_2O, water with all the PCR reagents. The experiment was repeated twice with independent RNA samples.

1999a). Both MCF-7 and MDA-MB-231 cells express functional β-adrenergic receptors and their signaling pathway through cAMP seems to be intact (Slotkin et al., 2000; Yasutomo et al., 1993). Several groups have shown, in different models, that the expression of D1 could be induced by different messengers or components exerting their effect through a cAMP pathway vgr, TSH in thyroid gland, carbohydrates in liver, NA in lactating mammary gland, etc. (Köhrle, 1997; Aceves et al., 1999a; Bianco et al., 2002). This effect can be blocked by transcription and translation inhibitors, indicating that persistent protein synthesis is required for the effect (Ishii et al., 1983; Pekary et al., 1994). However, the mechanism for the stimulation of D1 gene transcription by cAMP has not been clarified since the specific cAMP-response element (CRE) in the D1 promoter could not be identified. This D1 cAMP-mediated response disappeared in practically all carcinoma cells independently of the degree of cell differentiation and in spite of the presence of a growth stimulatory effect of TSH (Schmutzler et al., 1996; Schreck et al., 1994). This last data indicate a possible defect in the components of the signal transduction pathway of the TSH receptor different from those already characterized (Broecker et al., 1997) and suggest that absence of these responses could be one of the first alterations associated with tumorigenesis. Further studies are needed to elucidate the internal mechanism of these responses including the participation of T3, RA, and Es in their modulation, since evidence exists showing that all these components could modify specific D1 cAMP-mediated responses (Yasutomo et al., 1993; Pekary et al., 1994). Indeed, the co-transfected receptor models could be useful to analyze the participation of ovarian hormonal status in the responsiveness of RA and other agents in breast cancer.

In summary all these data indicate that the interrelation T3/RA/Es is an important component in the maintenance of cell differentiation and strongly support the proposal of D1 expression as a sensitive differentiation marker in cancer cells.

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